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PHENOMENON OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES: PASSIVE IMMUNITY TO REACTING FACTORS*

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The skin preparatory and reacting (*i.e.* intravenous) factors of the phenomenon of local skin reactivity to bacterial filtrates can be specifically neutralized by immune sera. The neutralization is accomplished by mixing *in vitro* the immune serum with the toxic filtrate (1-5). The object of the work reported in this paper was to determine whether it is possible to elicit passive immunity to the reacting factors.

EXPERIMENTAL

The *B. typhosus* toxic filtrates and antityphoid sera employed for this work were prepared according to the methods previously described (3, 6). Shortly before experiments were begun the reacting potency of the filtrates and the antireacting potency of the sera were determined (5). The titers are indicated in Table II.

In order to study the passive immunity to reacting factors the following was done:

The skin of rabbits was prepared by a single injection with 0.25 cc. of the toxic filtrate. After various intervals of time the prepared rabbits received an intravenous injection of serum followed by an intravenous injection of the toxic filtrate. The toxic filtrate was injected 24 hours after the skin injection of the filtrate. The interval of time chosen between the intravenous injection of the serum and the following intravenous injection of the filtrate varied, but in most experiments it was $\frac{1}{2}$ hour, in some experiments $1\frac{1}{2}$ and $2\frac{1}{2}$ hours.

* Read before the joint session of the American Association of Pathologists, Bacteriologists and Immunologists, Cleveland, April 2, 1931.

The results as summarized in Tables I, II, III and IV are as follows:

As is seen from Table I (Groups 2 and 3) only 16.6 per cent of rabbits passively protected by immune serum gave reactions and 90 per cent of non-protected rabbits (Group 1) showed reactions with a reacting dose even smaller than the one employed in the experimental group (dilution 1:10 in the control group and dilution 1:8 in the experimental group). Thus, it becomes at once evident that it is

TABLE I

Passive Immunity to B. typhosus Reacting Factors

Group No.	Serum	Amount of serum injected intravenously per kg. of weight	Toxic filtrate	Amount of serum injected intravenously per kg. of weight	Interval of time between intravenous injections	Order of intravenous injections	Total No. rabbits tested	No. negative rabbits	No. positive rabbits	No. of deaths
1	—	cc.	A-16 B. <i>typhosus</i> "agar washings" filtrate	1 cc. of 1:10 dilution	hrs.	—	10	1	6	3*
2	H ₄ Bl ₁₀₀ Antityphoid horse serum	3	" "	1 cc. of 1:8 dilution	$\frac{1}{2}$	Serum, filtrate	14	11	3	0
3	" "	6	" "	" "	$\frac{1}{2}$	" "	4	4	0	0

* Showing distinct local reactions.

possible to elicit passive immunity to *B. typhosus* reacting factors of the phenomenon of local skin reactivity.

Experiments on titration of protective potency of immune serum against *B. typhosus* reacting factors are reported in Table II. In these experiments antityphoid horse serum was injected intravenously into prepared rabbits $\frac{1}{2}$ hour prior to the intravenous injection of *B. typhosus* filtrate. Rabbits protected with various amounts of the

serum were tested against various numbers of toxic units. The largest number of toxic units giving no reaction in all rabbits tested was used to compute the *in vivo* potency of a given amount of serum. Thus, if 2 cc. of a certain serum injected intravenously, per kilo of body weight,

TABLE II
Passive Immunity to B. typhosus Reacting Factors

Group No.	Serum	Amount of serum injected intravenously per kg. of weight	No. of neutralizing units	Toxic filtrate	No. of reacting units injected intravenously per kg. of weight	Interval of time between intravenous injections	Order of intravenous injections	Total No. rabbits tested	No. negative rabbits	No. positive rabbits	No. of deaths	Ratio between <i>in vitro</i> and <i>in vivo</i> neutralization
4	H ₄ B1 ₁₀₂ Antityphoid horse serum	1 cc. of 1:4 dilution	125	A 53 B. typhosus "agar washings" filtrates	15	hrs. $\frac{1}{2}$	Serum, filtrate	3	1	2	0	—
5	" "	1 cc. 1:2	250	" "	20	$\frac{1}{2}$	" "	3	3	0	0	1:11
6	" "	1 cc. 1:2	250	" "	30	$\frac{1}{2}$	" "	6	3	3	0	
7	" "	2 cc.	1000	" "	40	$\frac{1}{2}$	" "	3	1	2	0	
8	" "	2 cc.	1000	" "	60	$\frac{1}{2}$	" "	3	3	0	0	
9	" "	2 cc.	1000	" "	80	$\frac{1}{2}$	" "	3	3	0	0	1:10
10	" "	2 cc.	1000	" "	120	$\frac{1}{2}$	" "	3	1	2	0	
11	" "	4 cc.	2000	" "	120	$\frac{1}{2}$	" "	3	3	0	0	1:11
12	" "	4 cc.	2000	" "	240	$\frac{1}{2}$	" "	3	1	2	0	
13	" "	6 cc.	3000	" "	500	$\frac{1}{2}$	" "	3	3	0	0	1:5
14	" "	6 cc.	3000	" "	700	$\frac{1}{2}$	" "	3	1	2	0	
15	" "	9 cc.	4500	" "	600	$\frac{1}{2}$	" "	3	3	0	0	
16	" "	9 cc.	4500	" "	900	$\frac{1}{2}$	" "	3	3	0	0	1:4.5
17	" "	9 cc.	4500	" "	1100	$\frac{1}{2}$	" "	3	1	1	1	

protected all rabbits tested against 80 units of filtrate and only some rabbits against 120 units, the protection titer of this amount of serum must be between these two figures or 100. Since the *in vitro* neutralizing titer of the sera was also determined, it was possible to derive the ratio between *in vitro* and *in vivo* neutralizations. The following conclusions can be drawn from the experiments recorded in Table II:

In Group 4: 1 cc. of 1 x 4 dilution capable of neutralizing *in vitro* 125 reacting units failed to give neutralization of as few as 15 units *in vivo*. The lack of neutralization can be attributed to a loss of antibodies in the general circulation. It is impossible to state whether the loss was partial or complete since rabbits protected with this amount of serum were not tested with a smaller number of reacting units. As the amount of serum injected was increased it became possible to demonstrate *in vivo* neutralization, decidedly less, however, than would have been obtained in direct *in vitro* neutralization. Thus, 2 cc. of serum injected per kilo neutralized 100 reacting units, that is, it was necessary to use ten times the number of neutralizing units as compared to *in vitro* neutralization with the same serum.

The experiments recorded in Table II also clearly demonstrated *in vivo* neutralization of reacting factors in multiple proportions. Thus, 250 units of serum protected against 25 reacting units; 4 x 250 serum units protected against 4 x 25 reacting units; 8 x 250 serum units protected against 7.2 x 25 reacting units.* However, 12 x 250 serum units protected against 24 x 25 reacting units and 18 x 250 serum units protected against 40 x 25 reacting units. As is seen from these figures the protection took place in direct multiple proportions until after a certain increase in the amount of serum injected, when there was a twofold and more than twofold gain in the protective value of the serum injected. In the interpretation of these facts of which the mechanism may be quite complex, there should be borne in mind a possible change in the rate of retention (*i.e.* lower elimination) of antibodies in the general circulation following the increase in the volume of serum injected. This explanation suggests itself at the present moment as the simplest one, but it remains to be proven.

The next point of interest was to determine the specificity of serum protection *in vivo* against *B. typhosus* reacting factors. In these experiments antimeningococcus immune horse serum of high neutralizing potency for meningococcus reacting factors and a normal horse serum were used. In view of the fact that *B. typhosus* antibodies are encountered in normal and heterologous immune horse sera (2), the

* This type of *in vivo* protection in multiple proportions is similar to *in vitro* neutralizations in multiple proportions of reacting factors. This question will receive special consideration in a separate report.

sera were also tested for agglutinins, precipitins and neutralizing antibodies. As is seen from the experiments recorded in Table III, antimeningococcus and normal horse sera in a dose of 4 cc. per kilo of body weight failed to protect against 40 reacting units, while the same

TABLE III
Specificity of in Vivo Neutralization of B. typhosus Reacting Factors

Group No.	Serum	Amount of serum injected intravenously per kg. of weight	<i>B. typhosus</i> (T ₁) agglutinin titer	<i>B. typhosus</i> (T ₁) filtrate precipitin titer	No. of antityphoid neutralizing units in serum per kg.	No. of <i>B. typhosus</i> reacting units injected intravenously per kg. of weight	Interval of time between intravenous injections	Order of intravenous injections	Total No. rabbits tested	No. negative rabbits	No. positive rabbits	No. of deaths
		cc.				units	hrs.					
18	Antimeningococcus horse serum H ₇ Bl ₁₈₇	3	N.dt.*	N.dt.*	N.dt.*	20	$\frac{1}{2}$	Serum, filtrate	6	1	4	1
19	Antimeningococcus horse serum H ₇ Bl ₁₈₉	4	1:64	0	240 units	40	$\frac{1}{2}$	" "	5	1	3	1
20	" "	4	1:64	0	240 units	60	$\frac{1}{2}$	" "	5	0	3	2
21	" "	7	1:64	0	420 units	40	$\frac{1}{2}$	" "	5	5	0	0
22	Normal horse serum	4	1:64	0	320 units	40	$\frac{1}{2}$	" "	4	0	4	0

* Not determined.

dose of antityphoid horse serum (Table II) completely protected against 180 reacting units. The normal and heterologous sera employed contained antityphoid antibodies, the concentration of which, however, in 4 cc. (*i.e.* 320 and 240 units, respectively) was insufficient to neutralize *in vivo* 40 reacting units, since, as described above, it is

necessary to have for this volume of serum eleven times more antibodies for *in vivo* than for *in vitro* neutralization. In Group 22, 7 cc. of antimeningococcus horse serum protected against 40 *B. typhosus* reacting units. It is clear that this protection could be easily attributed to the presence of antityphoid normal antibodies, since in 7 cc. there were present 420 neutralizing units, or more than ten times the amount required for *in vitro* neutralization. From these results it can be safely concluded that passive immunity to *B. typhosus* reacting factors is specific and depends on the concentration of homologous normal and immune antityphoid neutralizing antibodies.

TABLE IV

Protection of Rabbits after Injection of Reacting Factors

Group No.	Serum	Amount of serum injected per kg. of weight	No. of neutralizing units	No. of <i>B. typhosus</i> reacting units per kg. of weight	Interval of time between intravenous injections	Order of intravenous injections	Total No. rabbits tested	No. negative rabbits	No. positive rabbits	No. of deaths
23	H ₁₆ B1 ₁₉₄ Antityphoid horse serum	cc. 5	3000	50	None	Filtrate, serum	4	3	1	0
24	" "	6	3600	50	"	" "	4	4	0	0
25	" "	6	3600	75	"	" "	4	3	1	0
26	" "	6	3600	50	20 min.	" "	4	0	3	1
27	" "	6	3600	50	45 "	" "	4	0	3	1

It is also noteworthy that there may be observed a "prozone" effect of large amounts of serum. This is illustrated by the example of Group 7, Table II. The "prozone" effect in *in vitro* neutralization experiments was previously reported (4).

It was also attempted to determine the duration of passive immunity to *B. typhosus* reacting factors. The experiments demonstrated that the immunity established itself immediately after the intravenous injection of immune serum. From some experiments it appeared that half an hour after the serum injection the protection was more effective. No definite conclusions could be drawn, however, from these experiments since the "prozone" effect of serum pointed out above

could be a source of error. Indeed, under the given conditions, it was possible that during the half hour interval a part of the antibody was eliminated and thus the "prozone" effect was avoided.

Two experiments were carried out in which the interval of time allowed between serum and filtrate intravenous injections was $1\frac{1}{2}$ and $2\frac{1}{2}$ hours, respectively. Each of the rabbits received 3000 neutralizing units and 100 reacting units. The protection was complete in both groups. Longer intervals of time were not studied.

It was of considerable interest to determine whether the occurrence of the skin reaction could be prevented by serum injection if it followed the injection of the filtrate. Table IV represents the results obtained. As is seen from this table, it is possible to prevent the occurrence of the reaction by an intravenous injection of serum following the injection of reacting factors, provided the serum dose is very large and provided the injection of serum is made immediately after the filtrate injection. The skin reaction cannot be prevented if the immune serum is injected 20 minutes or longer after the injection of reacting factors. The results are most likely due to fixation of the reacting factors in the prepared tissue area shortly after their introduction into the general circulation. As a matter of fact, this can be expected, since even 1 hour after the intravenous injection of the toxic filtrate one can see a blue discoloration at the prepared skin sites, although it takes from 4 to 5 hours for the full development of hemorrhagic necrosis.

The results recorded in this paper also demonstrate passive serum protection against the lethal effect of *B. typhosus* culture filtrates in a ratio suggestive of multiple proportions. The protection is certain since control experiments (*i.e.* non-recorded titrations of potency of filtrates) showed consistently a high rate of mortality. It was already shown before that *in vitro* addition of immune serum to *B. typhosus* and other culture filtrates inhibited specifically their lethal effect (3).

DISCUSSION

In previous communications it was shown that the serum neutralization of reacting factors of the phenomenon of local skin reactivity is very similar to true toxin and antitoxin reactions. The neutralization is specific and follows the law of multiple proportions. The process of immunization stimulates formation of neutralizing antibodies, the

concentrations of which can be brought to high levels. Thus, in some horses, the serum containing normally from 10 to 20 neutralizing units showed from 175 to 200 units after 4 to 7 months of immunization. In other experiments, there was obtained by chemical means 28 to 80 times the concentration of these antibodies per 1 mg. of nitrogen (7).

When mixtures of filtrate and immune serum are made *in vitro* in order to carry out neutralization of reacting factors, there frequently forms an abundant precipitate. The precipitation may suggest that there is no actual neutralization of the toxic principle but a mere absorption of this principle by the precipitate. The experiments reported in the present paper, namely, *in vivo* neutralization of reacting factors in *multiple proportions* by means of immune serum, are considered as further evidence of the fact that the above discussed neutralizations are in the nature of toxin and antitoxin reactions.

The data presented thus far indicate clearly that a toxic, antigenic and neutralizable principle in bacterial filtrates is implicated in the phenomenon under discussion. The relationship of the principle to lethal factors present in the same filtrates remains to be determined. As described already, filtrates of agar culture washings prepared under conditions which would seem to allow of very little cell autolysis yield both powerful phenomenon-producing substances as well as highly lethal factors, the potencies of both being parallel to each other (8). Although rabbits show variations in susceptibility to the lethal effect of these filtrates, nevertheless the work carried out thus far on a very large number of animals brings unquestionable evidence that the lethal effect can be specifically abolished by immune serum. This observation was reported before in connection with *in vitro* neutralization experiments and is also definitely supported by the results recorded in this paper from which it is seen that the serum protection against mortality follows roughly the law of multiple proportions. It appears, therefore, suggestive that there are present in the filtrates prepared in the above described manner serum neutralizable lethal factors, which are associated with the phenomenon-producing factors. There is no experimental proof as to the identity of these factors.

The results on the neutralizability of bacterial lethal factors here recorded may appear to contradict the general belief that with the exception of a few true toxin producers, lethal factors produced by

the majority of microorganisms are only non-antigenic and non-neutralizable bacterial split products. For this reason the following points are of considerable significance in the evaluation of the results reported here:

1. A strain of *B. typhosus* (T_L) producing reacting factors of high antigenicity and neutralizability (5) was selected for this work.
2. Since the mode of preparation of the filtrates employed ("agar washings" filtrates) permits only insignificant cell autolysis, the results recorded here do not concern the lethal effect of bacterial split products.
3. It was shown by Ferry (9) and by Mishulow, Mowry and Scott (10) that the "agar washings" filtrates just mentioned are of high antigenicity.
4. Sera employed were of high neutralizing potency, as determined by the phenomenon of local skin reactivity.

CONCLUSIONS AND SUMMARY

It has proved possible to elicit passive immunity to *B. typhosus* reacting factors by means of normal and immune homologous neutralizing antibodies. The *in vivo* serum protection against these factors followed the law of multiple proportions.

There was observed a considerable loss of antibodies from the blood stream.

Passive immunity was best obtained when the immune serum was injected intravenously $\frac{1}{2}$ hour before the intravenous injection of the reacting factors.

It was possible to prevent the occurrence of the local skin reaction by an intravenous injection of serum after the intravenous injection of the reacting factors, provided the serum dose was very large and provided the serum injection was made immediately after the filtrate injection.

A number of experiments clearly demonstrated the interesting fact that the greater the amount of antiserum injected intravenously, the more efficient was the *in vivo* neutralization, in a ratio distinctly greater than the quantitative increase of serum. It is suggested that there may be a practical value of the observation in relation to serum therapy.

The results also demonstrated passive serum protection against the lethal effect of *B. typhosus* "agar washings" filtrates, in a ratio which seemed to suggest the law of multiple proportions.

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STUDIES ON TYPHUS FEVER

VIII. TICKS AS A POSSIBLE VECTOR OF THE DISEASE FROM ANIMALS TO MAN

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In his study of the epidemiology of typhus in the southern United States, published in 1926, Maxcy (1) came to a number of important conclusions, the most significant of which was that the epidemiological characteristics observed by him were at variance with the idea that the disease is kept alive by man to man transfer through lice. He suggested at that time, on the basis of case studies, that there might be an animal reservoir of infection, possibly in rodents—rats or mice—from which the disease is occasionally transmitted to man.

The most likely manner of such transmission, of course, would be by means of an insect, and in a previous study by the writers of this paper (2) experiments were reported on the infection of bedbugs with typhus virus, an insect which was investigated as the most likely possibility suggested by Maxcy's results. It was found at that time that bedbugs experimentally infected *per rectum* or permitted to feed on benzol-treated typhus rats could harbor the virus in potent form for from 5 to 9 days (the limits of the actual experiment). The viscera of such bedbugs injected into guinea pigs produced typical typhus infection with *Rickettsiae*, subsequent immunity to both European and Mexican typhus and characteristic brain lesions. We did not succeed in transmitting the disease to guinea pigs by the natural process of allowing the infected bedbugs to feed upon them. But the guinea pig is more resistant than man, and our cycle was in other respects complete in that we proved the possibility of natural infection of the insects and the viability of the potent virus within them for at least 9 days. These facts we took to be of considerable epidemiological importance

in view of the wide distribution of bedbugs and their plebeian catholicity in the choice of a large variety of hosts.

In the communication referred to above, Maxcy cites an observation made by Hone in Australia in which typhus fever occurred in individuals occupied in the handling of wheat and other food stuffs. He mentions another report, by Wheatland, in which typhus seemed related to a migration of mice and was spoken of as "mouse fever." Maxcy in his discussion concludes that rodents—rats and mice—constitute the most likely animal reservoir and that, as parasitic intermediaries, the first suspicions should fall upon "fleas, mites or, possibly ticks." The recent work of Dyer (3) has confirmed at least one of Maxcy's theories by the demonstration that rat fleas collected from rats trapped in a typhus focus contained a virus apparently identical with the "Wilmington" typhus strain of Maxcy.

It has thus been shown already that typhus virus can remain alive in at least three common, blood-sucking insects—the louse, the flea and the bedbug.

Our own success with bedbugs induced us to extend our studies to other insects before we knew of the work of Dyer and, for a number of reasons—the most potent of which was an extremely helpful letter from Dr. Halliday of the Maryland Department of Health—we chose ticks as our next line of attack.

Are Dogs Susceptible to Typhus?

Before beginning to work with the ticks themselves, we thought it important to determine whether or not dogs could harbor the typhus virus, since this might throw some light upon the type of tick with which it would be best to work. Dogs have no doubt been injected with typhus fever, but we could find no record of their having been injected with tunica rich in *Rickettsiae* from guinea pigs. We therefore injected two half grown mongrel puppies with a considerable dose of tunic suspension rich in *Rickettsiae*, a suspension which produced a rapid and typical disease in a control guinea pig, administering the virus intraperitoneally in one dog and intravenously in the other. One of these dogs, the intraperitoneally injected one, ran a temperature touching $104^{\circ}+$ on the 8th, 9th and 10th days, and the dog was for this reason killed and brain material injected into a guinea pig. The

other dog was observed for 21 days, during which he developed no febrile reaction, but brain from this dog was also injected into a guinea pig. Neither of these guinea pigs showed any symptoms or temperature simulating typhus fever, one of them running between 103° and 104°C. for about 2 or 3 days, but coming down again promptly. Both guinea pigs were subsequently tested for immunity and found susceptible.

We concluded, in consequence, that even large doses of infectious material did not produce either apparent or inapparent typhus in dogs, and that the dog is probably not, therefore, the animal reservoir for the disease. Further experiments on dogs of course should be done, but on the principle of following the most likely clue first, we abandoned work on dogs and turned our attention directly to the investigation of ticks that might be obtained from a variety of sources.

Experiments with Ticks

Experiment 1.—On Dec. 24, 1930, we obtained some ticks through the courtesy of Dr. Bustamente from Vera Cruz, which were later identified for us by Dr. Parker of the U. S. Public Health Service as belonging to the genus *Amblyomma*. On Jan. 12, 1931, eleven of these ticks were injected *per rectum* with the tunica emulsion of a guinea pig, material which was very rich in *Rickettsiae* and produced typhus fever promptly. The injection of the ticks was not easy. Some of them could be injected directly into the intestinal tract, but in the others the fluid passed into the celom. The injected ticks were incubated at 30°C. On Jan. 14th ten ticks which remained alive were put on a guinea pig for 24 hours' feeding. This guinea pig did not later develop typhus fever. We had in this first experiment an unfortunate mortality among the ticks, and on Jan. 21st only one of the injected insects remained alive. This tick was washed in alcohol, the viscera dissected out and injected into guinea pig Experiment 1, T 1. As seen in Chart 1 this guinea pig, after a preliminary rise of temperature, returned to normal, but on the 4th, 5th, 6th and 7th days ran a high temperature with early swelling of the scrotum. It was castrated on the 5th day and typical *Rickettsiae* found. The tunic of this guinea pig, T 1, was injected into T 2 and T 3 intraperitoneally with typical results, namely, temperature, swelling and *Rickettsiae* in both cases. It was carried into a third generation by injection of brain into Guinea Pig T 3 and tunica, and thence into a third generation, T 4 and T 5.

The accompanying chart of Guinea Pig T 1 and the protocol of subsequent manipulations of the typhus strain passed through the tick serve to describe this experiment.

Exp.1 G.P.T1

Amblyomma

Jan. 21, 1931

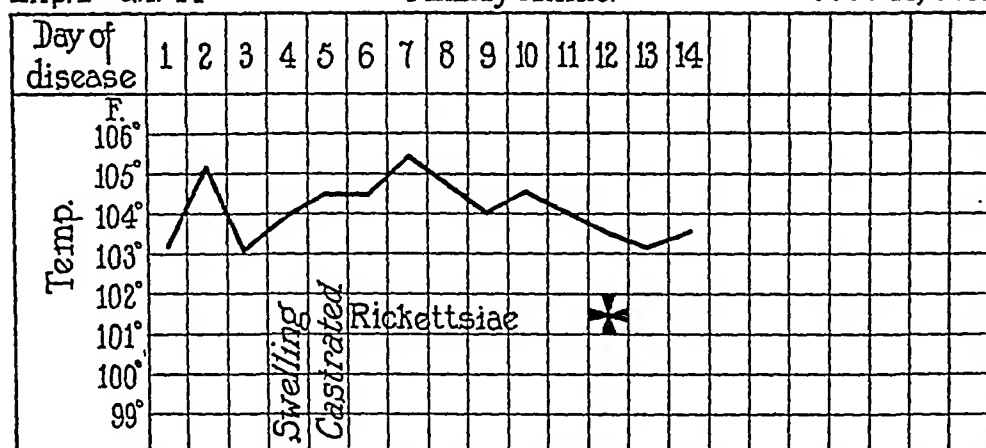


CHART 1. Record of guinea pig inoculated with viscera of *Amblyomma*, as described in Experiment 1.

Tick Experiment 1 (Protocol)

Guinea Pig 1

Typical Mexican typhus. *Rickettsia* material from tunica injected rectally into adult *Amblyomma* on Jan. 12, 1931

Jan. 21, 1931. Viscera of one surviving tick injected into *Guinea Pig T 1* (see Chart 1). Emulsions of tunica of this guinea pig injected into

Guinea Pig T 2

Swelling, 5th day. Temperature 105.6° on 6th day.
Castrated, 6th day. *Rickettsiae* +

Tunica 6th day into

Guinea Pig T 4

Temperature 105° and swelling, 6th day.
Rickettsiae +. Immune to European typhus fever

Killed and brain into

Guinea Pig T 5

Temperature 103.8° and swelling, 9th day.
Rickettsiae +

Guinea Pig T 3

Temperature 105° and swelling, 6th day.
Rickettsiae +. Immune to European typhus fever

Sections of brain of Guinea Pig T 2 showed no lesions, but T 3 and T 4, reinoculated with European typhus virus, simultaneously controlled on normal animals, proved to be immune.

Experiment 2.—*Dermacentor andersoni* adult ticks obtained through the kindness of Dr. Parker of the Hamilton, Montana, laboratory of the U. S. Public

Health Service were rectally injected on Feb. 26th by the capillary pipette method with tunica material from a Mexican typhus guinea pig, containing numerous *Rickettsiae*. These ticks stood the injections much better than those of the other species we had used, and very few died. Sixteen were injected and classified into those in which the rectal injection had been wholly successful and those in which success, as observed under the binocular, was questionable.

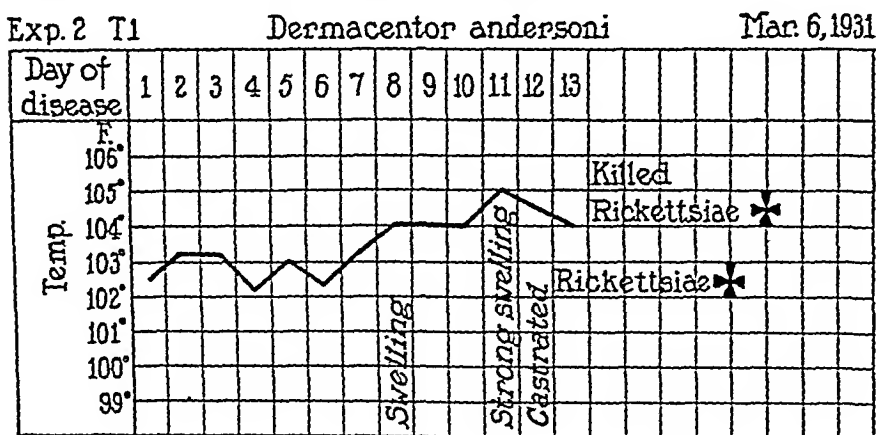
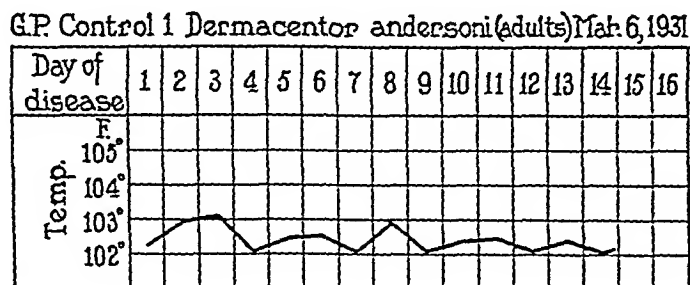


CHART 2. Reaction of guinea pig injected with *Dermacentor andersoni* viscera, as described in Experiment 2.



CONTROL CHART 2. Control guinea pig injected simultaneously with Chart 2 guinea pig with material from normal ticks of the same lot.

On Mar. 6th, 8 days after the rectal injection, three of these ticks were washed in alcohol, eviscerated as usual and the viscera injected into guinea pig Experiment 2, T 1.

At the same time, four ticks of the same lot, but uninfected, were similarly treated, and their viscera injected into a control guinea pig. The purpose of this

was twofold: First of all, to provide the control necessary to insure against error from unknown causes, but also to make sure that the ticks as received did not naturally harbor either Rocky Mountain spotted fever or tularemia. Dr. Parker had called our attention to these possibilities, stating that he thought them unlikely, but advised caution.

The guinea pig injected with infected ticks developed a temperature of 104°F., with scrotal swelling, on the 8th day. By the 11th day the swelling was much increased and the temperature had risen to 105°. At this point one testicle was removed and plentiful and typical *Rickettsiae* found in the tunica. On the 13th day the animal was killed and *Rickettsiae* again found in smaller numbers. The brain was removed, sectioned and a few small lesions found which Dr. Wolbach was kind enough to examine for us and which he regarded as consistent with early typhus brain lesions. In view of the fact that the animal was killed early, and that Mexican typhus animals do not show brain lesions with the same regularity and number as do those infected with European virus, these lesions, though small and few, have considerable importance.

The control guinea pig injected with the viscera of the uninfected ticks of the same lot remained normal.

These results are shown in Chart 2, under the headings of Experiment 2, T 1 and Guinea Pig Control 1. The subsequent course of the experiment is discussed in the protocol.

Tick Experiment 2 (Protocol)

Feb. 26, 1931, *Dermacentor andersoni* rectally injected with Mexican typhus virus. Mar. 6th, into

Guinea Pig T 1

Temperature and swelling, 8th to 12th day. *Rickettsiae* +.

Killed 13th day—brain lesions, few and small but suggestive.

March 13th—tunica material of 8th day into

Guinea Pig T 2

Swelling and temperature 105° 5th day.

Rickettsiae +. Reinoculated on 13th day and found immune

Guinea Pig T 3

Swelling and temperature 105.6° on

4th day. Castrated. *Rickettsiae* +. Tunica material into

Guinea Pig T 4

Typical temperature and swelling, 4th and 5th days

Experiment 3.—Two of the same ticks used in Experiment 2 and injected interrectally on Feb. 26th were allowed to feed on a normal guinea pig from Mar. 6th to Mar. 9th by the method formerly used by Dr. Wolbach, namely, attaching them in an open pill box with a bandage to the shaved abdomen of a normal animal. They were found containing some blood and dug into the skin of the guinea pig

on Mar. 9th and induced to let loose by snipping the skin just in front of the imbedded sucking apparatus. This was done because we had been told by Dr. Parker that infected ticks become more potent in virus after feeding. From Mar. 9th up to Mar. 12th they were kept at 30° in an incubator. On Mar. 12th, 14 days after intrarectal injection, two ticks treated as above were washed in alcohol, eviscerated and the viscera injected into the guinea pig labelled

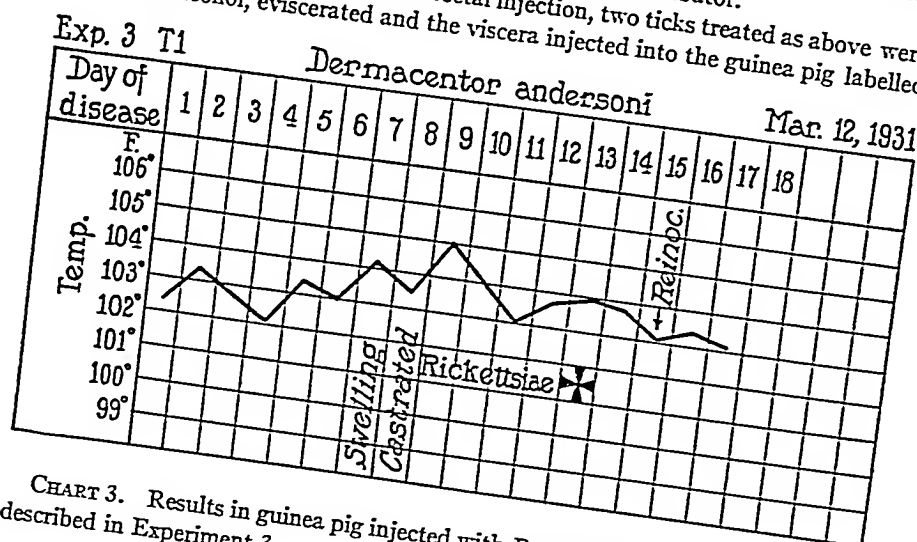
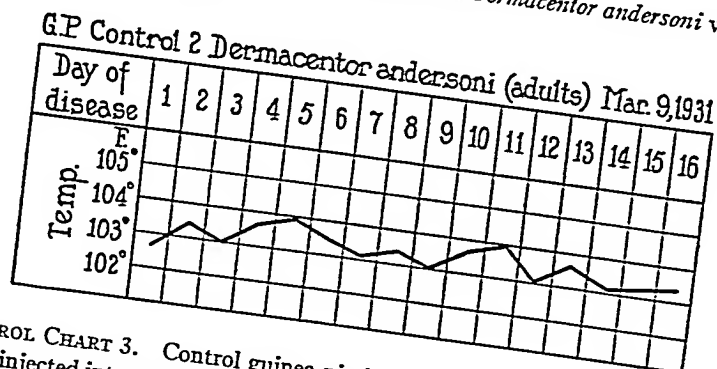


CHART 3. Results in guinea pig injected with *Dermacentor andersoni* viscera, as described in Experiment 3.



CONTROL CHART 3. Control guinea pig injected with normal ticks of same lot as those injected into guinea pig on Chart 3.

Experiment 3, T 1. A control of the same lot of ticks, in which the viscera of four uninfected insects were used, was done on Mar. 9th.

The guinea pig inoculated with the two infected ticks treated as described above showed a temperature of 104° on the 7th day, with typical swelling, and was castrated. Plentiful *Rickettsiae* were found in the tunic.

The temperature of this animal dropped to 102° on the 15th day, when it was reinoculated with Mexican typhus virus and found immune, controls of course being done with the same virus.

The subsequent passages of this tick virus is indicated in the following protocol.

Tick Experiment 3 (Protocol)

Dermacentor andersoni adults infected rectally with tunica material on Feb. 26th.

2 of these ticks fed for 2½ days on normal guinea pig, Mar. 6th to Mar. 9th.

Kept at 30°C. until Mar. 12th. Viscera injected into

Guinea Pig T 1

(See Chart 3, Experiment 3, T 1. Temperature and swelling on 7th day.

One testicle removed on 9th day. *Rickettsiae* +. Reinoculated on 15th day and found immune. Tunica of 9th day into

Guinea Pig T 2

Swelling and temperature 105° on 6th and 7th days

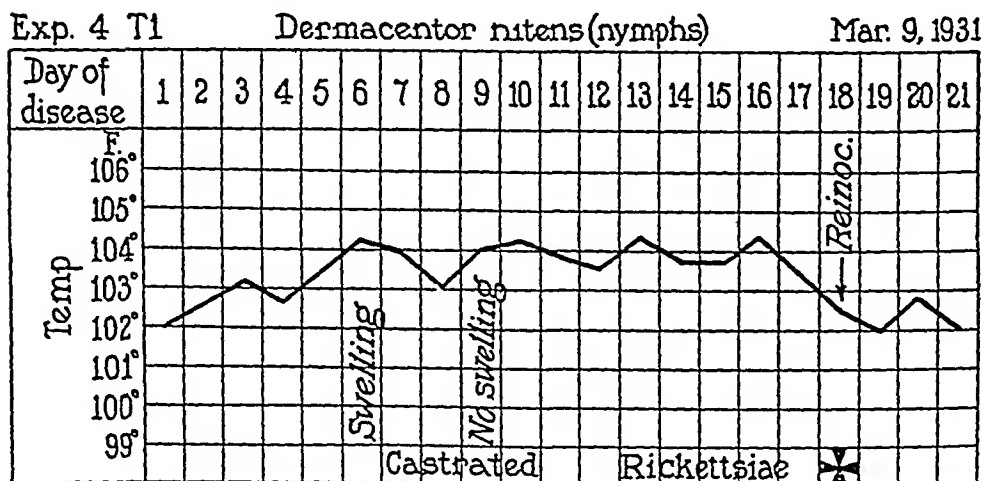


CHART 4. Reaction in guinea pig inoculated with viscera of *Dermacentor nitens* nymphs, as described in Experiment 4.

Experiment 4.—We had received through the kindness of Dr. Iglesias a number of ticks of two varieties from the neighborhood of Vera Cruz. Some of these were *Amblyomma*, others were nymphs of *Dermacentor nitens*. Unfortunately, many of these ticks were adult females which were in the process of laying eggs and died in the course of unsuccessful experimentation, most of them dying within a few days after intrarectal injection. The only insects of this lot that were successfully handled were two *Dermacentor nitens* nymphs which were injected intrarectally

with Mexican typhus virus on Feb. 27th, and whose viscera were inoculated intraperitoneally on Mar. 9th into a guinea pig charted as Experiment 4, T 1. This guinea pig developed on the 6th day typical swelling and a temperature of 104.2° . One testicle was removed on that day and *Rickettsiae* typical in appearance and position were found. The temperature continued for 16 days, the swelling subsiding. On Mar. 27th this animal was reinoculated with Mexican typhus virus and found immune. See Chart 4.

The subsequent course of this experiment is described in the following protocol.

Tick Experiment 4 (Protocol)

Two *Dermacentor nitens* nymphs were rectally injected with typhus virus on Feb. 27th, 1931. Kept at room temperature. On Mar. 9th, eviscerated and viscera injected into

Guinea Pig T 1

Swelling and temperature 104.2° on 6th day. One testicle removed. Plentiful *Rickettsiae*. Reinoculated, and found immune on Mar. 24th. Material of removed testicle inoculated into

Guinea Pig T 2

Typical disease. *Rickettsiae* ++.
Immune on reinoculation

Guinea Pig T 3

Typical disease. *Rickettsiae* ++
Immune on reinoculation

Four control guinea pigs into which the viscera of presumably normal ticks had been injected without arousing reactions were subsequently inoculated with typhus virus and found susceptible.

Not all attempts to pass the virus through ticks were successful. In addition to the positive experiments above recorded, failures should be reported as follows:

1. Two *Dermacentor* adults were intrarectally injected with virus on February 28th, 1931. Their viscera inoculated into a guinea pig on March 9th produced neither infection nor immunity.
2. Two *Dermacentor* adults treated exactly as were the two described in Experiment 2, except that feeding on a normal animal was not carried out in the interval between rectal infection and visceral inoculation, likewise were negative. This may have some importance in that Dr. Parker tells us that infected Rocky Mountain spotted fever ticks may increase in virulence after feeding.
3. *Dermacentor andersoni* nymphs allowed to feed on typhus infected and benzolized rats did not produce typhus fever on guinea pig injection 6 and 14 days respectively after the last date of feeding.

All but one of the guinea pigs unsuccessfully inoculated in this manner were subsequently infected with typhus virus and reacted typically, thus adding further controls to show that the normal ticks used by us contained no virus that could either simulate typhus fever in the guinea pig or produce immunity.

DISCUSSION

Our experiments have shown that the virus of Mexican typhus fever rectally injected into ticks of three varieties (*Dermacentor nitens*, an *Amblyomma* not further identified and *Dermacentor andersoni*) will remain alive and potent in these insects for as long as 14 days, the limit of our experiments.

Guinea pigs injected with the viscera of such ticks develop the temperatures, scrotal swellings and tunica lesions characteristic of Mexican typhus infections. Typical and plentiful *Rickettsiae* were always found, brain lesions—though few and small—were seen in one of two animals examined, and six guinea pigs so infected were found immune on subsequent inoculation with the virus itself in amounts which caused severe and characteristic reactions in control animals.

These experiments, added to Nicolle's fundamental discovery regarding lice, Dyer's observations on rat fleas and our own work with bedbugs, make a fourth group of blood-sucking insects which can harbor the virus of typhus fever. Our work, it is true, was carried out by experimental injection and not by the natural methods of infection and transmission by feeding. It does not, therefore, justify the deduction that ticks can become typhus vectors in the course of their natural existences. The experiments, however, render this a distinct possibility. That the louse transmits typhus fever from man to man under epidemic conditions and, perhaps in most regions in endemic cases, is, of course, unquestionable in the light of the classical investigations of Nicolle and his collaborators (4), of Ricketts and Wilder (5) and innumerable subsequent students of the disease. That the louse is not likely to account for the sustained endemic prevalence of the disease in regions like the southern United States has been pointed out by Maxcy.

Maxcy's suggestion that there is an animal reservoir has been upheld by the rat flea observations of Dyer. The fact, however, that the

virus can survive in a number of widely differing insects gives rise to the thought that the flea may not be the only possible vector.

Ticks can take blood from a variety of animals associated with man; they are widely distributed, at least in American and Mexican typhus regions; they have suggested themselves as possible transmitting agents to those who have made epidemiological studies of the American disease (Maxcy (1), Halliday, personal letter), and they convey the closely related disease, Rocky Mountain spotted fever. Our experiments add another fact in favor of the possible incrimination of ticks, together with bedbugs and fleas, in the epidemiological cycle which keeps typhus fever prevalent in places and at periods in which man to man transmission is unlikely.

The viability of the virus in ticks naturally leads to the thought that dogs may represent the animal reservoir. Two experiments with dogs failed to show that these animals could either contract the disease or harbor the virus in an "inapparent" form. Rats and mice, therefore, in view of Dyer's work, are the most likely animal sources.

CONCLUSIONS

Mexican typhus virus can be passed through ticks by the method of rectal injection.

The virus will remain alive in the ticks for at least 12 days.

These studies, together with one of our preceding publications and the work of Dyer, demonstrate that there are at least three insects—bedbugs, fleas and ticks—which must be considered as possibilities in conveying typhus fever from an animal reservoir to man. Our work will be continued by a study of rats and mice caught in typhus regions such as Mexico City and its immediate vicinity, with a search for the virus in these rodents as well as an analysis of the insects found upon them or in the localities in which they are concentrated.

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EXPERIMENTAL HYPERTHYROIDISM AND ITS EFFECT UPON THE MYOCARDIUM IN GUINEA PIGS AND RABBITS

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PLATE I

It has been known for some years that the administration of preparations of the thyroid gland gives rise to an hypertrophy of several organs including the heart. Iscovesco (1, 2), using rabbits, produced these changes by daily injections of a "lipoid" extract. Hoskins (3), Herring (4), Hewitt (5), and others fed rats daily on various preparations of the gland and noted that, even when the doses used were "non-toxic" (*i.e.*, failed to produce evidences of hyperthyroidism), cardiac hypertrophy resulted. Cameron and Carmichael (6) studied rats and rabbits with similar results; Simonds and Brandes (7) fed dogs large quantities of dried thyroid (10 gm. and more) daily, so that definite hyperthyroidism was produced, and found considerable increase in the size and weight of the hearts. Observers agree that hypertrophy so produced involves all chambers of the heart with a slightly greater proportional increase in size of the left ventricle; it tends to disappear when the thyroid preparation is discontinued.

The histological changes in the heart resulting from induced hyperthyroidism have not been studied so thoroughly and the results are conflicting. Farrant (8) examined the hearts from cats and rabbits that had been fed various thyroid preparations and described wasting of the muscle fibres or an hyaline appearance of individual fibres; there was no cell infiltration. On the whole, his meagre histological account fails to show convincingly that any pathological changes were produced. Cameron and Carmichael found no histological changes in the hearts of their rats. Hashimoto (9) made an extensive study of the changes in rats' hearts. Thirty-eight animals were fed on large doses of thyroid (0.1 to 0.5 gm. daily). Of these, ten died and the rest were killed with chloroform (see Goodpasture below). 90 per cent of those fed 0.5 gm. daily and 50 per cent of those fed 0.3 daily showed diffuse myocardial changes characterized by swelling and separation of the fibres, and dense patches of small round cells, monocytes, and a few fibroblasts. These lesions appeared for the most part close to blood vessels and

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under the pericardium; they were said to resemble Aschoff bodies. Four of eleven animals given 0.03 gm. every other day showed similar lesions. Nothing comparable was found in any one of the thirty-six control animals. Hashimoto believed that he had produced in his rats lesions similar to those described by Fahr (10) in hyperthyroidism in man.

In the same year, and entirely independently, Goodpasture (11) published the results of a similar study on rabbits. Seven animals were fed dried thyroid gland (1 gm. daily for 15 to 26 days), and a like number were injected intravenously with thyroxine (1 mg. every 2nd or 3rd day for 9 to 23 days). The animals were killed by a blow on the head. The auricles showed no changes. Small "definite but insignificant" changes were found in the ventricles, consisting in perivascular fibrosis and slight destruction of fibres about the small vessels in the wall of the right ventricle, with fibrosis in the wall of the left ventricle and papillary muscles. One animal, which died as a result of the hyperthyroid state, showed small fresh necroses. Fifteen animals treated in the same manner were, in addition, subjected to chloroform inhalation for 1 hour and killed 24 to 72 hours later. Twelve of these animals showed areas of necrosis in the left ventricle, mononuclear infiltration, and later the appearance of fibroblasts. Goodpasture concluded that large doses of thyroid products produced the symptoms of hyperthyroidism, but caused only insignificant histological changes in the heart. On the other hand, he believed that these products rendered the myocardium more susceptible to injury, since chloroform inhalation, which in ten control animals had failed to produce any myocardial lesions, produced necroses in the hyperthyroid animals.

Takane (12) fed thyreiodin to rats, guinea pigs, and rabbits. He found that when large quantities were avoided (*i.e.*, 0.08 gm., which caused too rapid death), an acute myocarditis was produced with changes in the muscle fibres, and a diffuse or localized infiltration of cells in the wall of the left ventricle. The changes appeared about the 7th day. Sodium and potassium iodides produced similar changes. From the figures of von Furth (13) and Riesenfeld (14), Takane assumed that the heart contained more lactic acid than any other organ, and that this substance liberated free iodine which produced the myocarditis. This explanation is often accepted and frequently quoted, but the figures themselves are open to question. Von Furth's estimations were made on tissues 48 hours post mortem, Riesenfeld's following sudden death in one individual. Subsequent work on animals, with more rapid and careful technique, has shown that the lactic acid values of resting mammalian skeletal muscle are more than double those of the myocardium (15, 16).

The lack of uniformity in the microscopical findings of induced hyperthyroidism is striking. Hence we have undertaken to determine what these are. Cameron and Carmichael found no changes. Those described by Farrant and Goodpasture are insignificant, while those of Hashimoto and Takane are apparently well marked and extensive.

Hashimoto's use of chloroform to kill twenty-eight of his thirty-eight rats suggests, upon first thought, that he may have accidentally reproduced Goodpasture's experiments. Since however, considerable time would be required for the appearance of the small round cells, monocytes, and fibroblasts found in the lesions such an inference seems improbable.

Material

In the present work both rabbits and guinea pigs were used. The weight, temperature, and heart rate of each animal was recorded every other day during the experiment. The injections of thyroxine were made intramuscularly on alternate days; the quantities varied slightly. Guinea pigs received thyroxine in doses of 0.10 to 0.17 mg. per day over 4 to 83 days. Rabbits received doses of 0.10 to 0.35 mg. per day over 2 to 13 days. Thyroxine ("Roche") was used throughout. The degree of hyperthyroidism produced was judged by the loss of weight, the increase in heart and respiratory rate, diarrhea, apprehensiveness, and, in more extreme instances, by the great muscular weakness. It was observed that the animals showed great individual variation in susceptibility to thyroxine. Evidences of hyperthyroidism appeared as a rule within 36 to 48 hours after the first injection, and progressed with succeeding doses. Of the animals which died, all were discarded except those that were still warm when found. The majority of animals were killed by a sharp blow on the head. The tissues were fixed almost immediately in either Zenker-formol solution or in formalin. Sections were made from both auricles and ventricles and in practically every instance from the lungs and adrenals also.

In the case of both rabbits and guinea pigs, a series of control (normal) hearts were examined, in order to ascertain the variety and extent of changes present in normal animals.

The Findings in Guinea Pigs

The hearts from twenty normal guinea pigs were carefully studied. These animals were taken from stock, weight and temperature were recorded on alternate days over periods of time corresponding to the experiment, and if any animal showed signs of ill health it was discarded.

In the majority of control hearts, there were vacuolated muscle fibres which were more plentiful beneath the epicardium and endocardium. Beneath the endocardium, especially close to the point of attachment of the valve cusps, cells were abundant, either scattered or in small groups. These cells were for the most part monocytes or lymphocytes, but there were also occasional pseudo-eosinophil leucocytes. Less conspicuous were small groups of monocytes and lym-

phocytes close under the pericardium and in association with the smaller branches of the coronary arteries. Finally, occasional collections of similar cells were seen between the muscle fibres. The fibres themselves showed no changes apart from the vacuoles already mentioned. There was no necrosis nor any scarring.

Complete autopsies were performed on seventeen guinea pigs, which had received thyroxine. Eight of these showed evidence, either macroscopic or microscopic or both, of spontaneous pneumonia apparently with *B. bronchisepticus*; the other nine showed no infection. For reasons which will shortly be obvious, these two groups will be discussed separately.

In the nine uninfected animals no myocardial changes whatever were found save in No. 17. In one place in the wall of the left ventricle of this animal there was found a small area of dead muscle fibres surrounded by a large number of lymphocytes; there was no other abnormality. These nine animals received injections of thyroxine varying from 0.07 to 0.17 mg. daily over periods up to $10\frac{1}{2}$ weeks, and in six of them very severe hyperthyroidism was produced. Singularly enough, the one animal to show a lesion was the one with the least evidence of hyperthyroidism. It was noted in both guinea pigs and rabbits that the hearts of hyperthyroid animals were considerably larger than the normal. This appeared to be due largely to dilatation, though doubtless some hypertrophy was present also. The heart invariably stopped in diastole, leaving the chambers dilated and the auricles filled with dark blood.

Rigor mortis of the skeletal muscles occurred much sooner after death in the hyperthyroid than in the normal animals, though neither were allowed to struggle. Hashimoto (9) noted this phenomenon in the cardiac muscle of his animals.

Of the eight infected guinea pigs (*bronchisepticus*), five showed definite myocardial damage. The changes were variable. In places there were small masses of necrotic, granular, and pink staining tissue surrounded by monocytes and pseudo-eosinophil leucocytes (Fig. 1). In other places a few cells were collected around one or two dead and granular muscle fibres. At a later stage, there were seen occasionally areas of vacuolated and shrunken fibres with collapsed stroma, a few lymphocytes, and some fibroblasts. Another lesion was char-

acterized by the accumulation of large clear elongated cells, with clear nuclei, which surrounded degenerated muscle fibres, often in association with small vessels. On more detailed examination these cells showed a very close resemblance to the cells of the sarcolemma, and it is believed that they represented a proliferation of those cells, perhaps in an attempt at repair. An extreme example of such accumulation of large clear cells is seen in Fig. 2. Finally, in two cases there was extreme vacuolation of the muscle fibres so that they appeared to be "blown out" (Fig. 1). These vacuoles did not stain with fat stains.

It seems certain that these lesions are not due to the hyperthyroidism alone, but it is conceivable that, as in Goodpasture's chloroform experiment, the hyperthyroidism may have had an adjuvant action. From the meagre literature on spontaneous *bronchisepticus* infection it is difficult to ascertain what damage this disease can produce in the myocardium. The most definite statement on the subject is in the paper by Strada and Traina (17), describing an epidemic of pneumonia in guinea pigs, caused by an organism which subsequent investigators (18) have recognized as *B. bronchisepticus*. They say that the organ next most affected after the lung itself is the heart, which often shows well marked areas of "degeneration."*

The Findings in Rabbits

Forty-three control "normal" rabbits were taken from stock and killed after a period of observation similar to that of the guinea pigs. In each case a complete autopsy was performed and the myocardium examined with especial care. As in the guinea pigs, the majority of normal hearts showed vacuolated muscle fibres. Occasionally this change was extensive and well marked. Groups of lymphocytes and monocytes were present close to the small vessels and between the muscle fibres. Most striking were the occasional areas of fibrocytes and lymphocytes associated with vacuolated and collapsed muscle fibres (Fig. 3). In other cases there were less conspicuous areas in which

* Oskar Seifried (in Jaffé's recently published *Anatomy and pathology of spontaneous diseases of small laboratory animals*, Berlin, 1931, 584) says of pleuropneumonia due to *B. bronchisepticus*, "moreover degeneration of the myocardium, congestion, and cloudy swelling of the parenchyma are often demonstrable."

a few lymphocytes were accumulated around disintegrating fibres and collapsed stroma. Areas of scarring were sometimes found, most frequently in the base of the papillary muscles, and in one case there was a collection of large clear cells, resembling those of the sarcolemma, close to a small vessel. In all, six out of the forty-three normal controls showed the definite abnormalities described—focal destruction of muscle fibres and cell infiltration.

Forty-four rabbits, which received intramuscular injections of varying amounts of thyroxine every other day, were killed and complete autopsies performed. Of these forty-four rabbits, fifteen showed lesions in the myocardium. Some of these lesions were obviously fresh; small groups of necrotic and disintegrating fibres were surrounded by lymphocytes, monocytes, or more rarely pseudo-eosinophils (Fig. 4). At a later stage there were areas from which the fibres had largely disappeared, the stroma had collapsed, and fibroblasts were present. Finally there were distinct and dense scars. Apart from this type of lesion, three of the cases showed greatly vacuolated or blown out muscles fibres similar to those noted in the guinea pigs, and, in two cases, areas of what were apparently proliferated sarcolemma cells were seen. On the whole the lesions in the myocardium of these rabbits differed only in degree, and that slightly, from those seen in the control series.

DISCUSSION

In the case both of guinea pigs and rabbits the administration of thyroxine alone has produced, at most, only insignificant morphological changes in the myocardium. In the series of uninfected guinea pigs, only one out of nine showed a myocardial lesion, and it is possible that this animal, No. 17, may in reality have had an early infection in one lung which the single routine section failed to show. The relation of this single lesion to the hyperthyroidism seems improbable, since, as has already been pointed out, this guinea pig showed the least evidence of hyperthyroidism of any animal in the series. It is obvious that the frequent occurrence of myocardial lesions in the animals with pulmonary infection is of considerable importance in the interpretation of results. Inasmuch as previous investigators have not dealt, specifically, with this point, it is difficult to evaluate the

significance of the myocardial changes which they have described. In the present study, animals placed in the control group and given no thyroxine were discarded if infections occurred. It is not known therefore whether cardiac lesions, similar to those described above, occur as a result of infections in the absence of hyperthyroidism.

The lesions produced in the rabbits were different only in degree, and not in quality, from those seen in the controls. This latter point is interesting, and indeed may serve as a clue towards the solution of the problem. As has been pointed out elsewhere (19), the lesions in the myocardium of human cases of hyperthyroidism differ in degree only from the changes found in a control group of "normal"* hearts of the same age period. It would seem probable, on the basis of this evidence, together with that presented in the present paper, that the hyperthyroidism itself produces no specific lesion. It is conceivable that damage produced, on the one hand, by the wear and tear and on the other, by any associated infection or other disease, tends to be more accentuated in the individual with hyperthyroidism than in the normal individual. This point is however far from settled.

It is certain that in the past too much emphasis has been laid upon the morphological changes in the myocardium, with a consequent neglect of important alterations in the metabolism and function of the cardiac muscle. It would seem probable that physiological studies may bring us to a better understanding of the cardiac abnormalities in hyperthyroidism.

SUMMARY

A study has been made of the pathological changes in the hearts and other tissues of animals rendered hyperthyroid with thyroxine. Forty-four rabbits and seventeen guinea pigs were given intramuscular injections of thyroxine every other day and sacrificed at varying intervals. Tissues from a series of normal animals (twenty guinea pigs and forty-three rabbits) were examined as a control. The changes in the heart and other tissues of hyperthyroid animals were insignificant and varied but little from changes seen in normal control animals. Of eight thyrotoxic guinea pigs that developed coincidental infection

* These normal hearts were chosen from individuals who had no cardiac disease nor evidence of any specific or unspecific infection.

(*bronchisepticus*) all showed myocardial lesions. Of nine thyrotoxic guinea pigs, free of infection, only one gave evidence of myocardial change. It is pointed out that hyperthyroidism, *per se*, cannot be held responsible for these lesions, which would appear to have been associated with the infection.

It was noted that rigor mortis of the skeletal muscles occurred much sooner in the bodies of hyperthyroid animals than in normal animals.

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EXPLANATION OF PLATE 1

FIG. 1. Section of myocardium from hyperthyroid guinea pig infected by *bronchisepticus*. Area of necrotic fibres surrounded by leucocytes. Vacuolation of muscle fibres. $\times 94$.

FIG. 2. Section of myocardium from infected, hyperthyroid guinea pig. Accumulation of large clear cells and of leucocytes. $\times 94$.

FIG. 3. Section of myocardium from a normal rabbit. Muscle fibres replaced by area of fibroblasts and lymphocytes. $\times 112$.

FIG. 4. Section of myocardium from an hyperthyroid rabbit. Area of disintegration of fibres with small round cells. $\times 112$.



(Rake and McEachern: Effect of hyperthyroidism on myocardium)

monkeys characteristic granular conjunctivitis—a finding similar to that of Finnoff and Thygeson (1) with materials obtained from white patients in Denver.

Methods and Materials.—There were available for study nine patients residing in New York City, all having advanced trachoma of from 1 to 11 years' standing. With two exceptions (Cases 7 and 8), the patients exhibited scar tissue and characteristic pannus formation. The materials used consisted of the affected conjunctivae removed, for curative purposes, from the novocaine-anesthetized upper lid. We are greatly indebted to Dr. Martin Cohen² for these materials, and for other aid. The fresh tissue from each of eight of the cases was ground in saline solution and inoculated subconjunctivally into the left upper lid of two or three *Macacus rhesus* monkeys, using methods of injection already described (2, 3). In another series of tests, the conjunctival secretions from each of two patients were used as inocula. The excised conjunctiva of one of the latter patients was used for subconjunctival inoculation of monkeys, 19 days after the final collection of secretions. The secretions were taken on cotton swabs and, by gently rubbing the mucous membrane, were transferred directly to the conjunctivae of each of three monkeys. Nine swabbings at daily intervals were made with the secretions of the first case and seven with those of the second.

Only such monkeys as had smooth conjunctivae at the end of an isolation period of about 1 month were employed, so as to decrease the hazard of confusion with spontaneous folliculosis.

*Results of Animal Inoculation.*³—The results obtained after single subconjunctival inoculations of monkeys with suspensions of conjunctival tissue obtained from man will be considered first.

Case 1.—Male, 26 years old, Russian. Has had trachoma for 10 years. Scars and pannus present. Suspension of conjunctival tissue inoculated into the left conjunctiva of each of three *rhesus* monkeys (A, B, and C). All these animals showed the characteristic granular conjunctivitis described by Noguchi (3) as following inoculations of *Bacterium granulosis*. For example, Monkey A revealed follicles in the conjunctivae of both eyes on the 11th day after inoculation, and Monkeys B and C reacted similarly 4 days after the injection.

Case 2.—Female, 20 years old, Italian. Has had trachoma for several years. Scar tissue and pannus present. Monkeys D and E injected with suspension of patient's conjunctival tissue. Monkey D showed characteristic granular conjunctivitis in the left eye 33 days after the injection and in both eyes 41 days thereafter. Monkey E remained unaffected.

² We are also grateful to Drs. Arnold Knapp, Ervin Torok, and Julius Wolff, all of New York City, for their cooperation.

³ All operations were done under full ether anesthesia.

Case 3.—Male, 26 years old, American. Duration of trachoma, 7 or 8 years. Pannus and scars present. Monkeys F and G injected with tissue. Monkey F revealed the first signs of the characteristic experimental conjunctivitis in the left eye 10 days, and in the right, 45 days after inoculation. Monkey G failed to show lesions.

Case 4.—Male, 29 years old, German. Trachoma of uncertain duration, probably several years. Pannus and scars in left eye, scars only in right. Tissue from both upper lids pooled and injected into monkeys H and I. Monkey H developed the characteristic experimental disease in the left eye 7 days after inoculation. It died of tuberculosis, however, 1 month later. Monkey I showed granular conjunctivitis in both eyes also after 7 days.

Case 5.—Female, 50 years old, Russian. Duration of disease 10 years. Scar tissue and pannus present. The right and left upper conjunctivae were removed and a suspension of both tissues pooled was inoculated into the left conjunctiva of each of two monkeys, J and K, neither of which was affected by the injection.

Case 6.—Male, 48 years old, Italian. Duration of trachoma, 3 years. Scar tissue and pannus present. Tissue injected into two monkeys, L and M. Neither showed lesions.

Case 7.—Male, 31 years old, American. Has had trachoma for 11 years. 10 years ago, left tarsectomy performed and 5 years ago expression of follicles on left conjunctiva. The right upper conjunctiva showed large follicles, but no scars or pannus. A few follicles were also noted in the lower membrane and in the residual tissue of the left eye. Suspension of the upper right conjunctival tissue was inoculated into the left conjunctivae of each of two monkeys, N and O. Monkey N developed characteristic experimental conjunctivitis in both eyes 13 days after inoculation. Monkey O showed follicles in the left conjunctiva 6 days after injection, and in the right, 8 days later.

Case 8.—Male, 21 years old, German. Duration of disease 1 year. Treated for 2 months prior to tarsectomy. No scars or pannus present. Two monkeys, P and Q, inoculated subconjunctivally with suspension of patient's conjunctival tissue failed to become infected.

The next series of tests concern the production of the experimental disease in *Macacus rhesus* monkeys by means of swabbing the secretions of the patients on to the conjunctivae of the animals.

The first experiment was made with secretions obtained from Case 1, already mentioned, the secretions having been collected 19 days before the tarsectomy was performed. 13 days after the ninth and last swabbing, Monkey R developed the characteristic granular conjunctivitis; Monkey S died of tuberculosis during the swabbings; and Monkey T was unaffected.

The secretions for the second test were obtained from Case 9.

Case 9.—Male, 28 years old, Italian. Had trachoma for 2 years. Scars and

pannus present. 13 days after the seventh and last swabbing with this patient's secretions, three monkeys (U, V, and W) presented typical granulomatous changes in their conjunctivae. In none of the three animals, however, was extension of the affection noted in the untreated conjunctiva.

To summarize, a characteristic infection was produced in *Macacus rhesus* monkeys, either by means of a single subconjunctival injection of suspensions of conjunctival tissue from human trachoma, or by means of conveying the patient's secretions with cotton swabs to the animal's conjunctiva. By the former method, five out of eight patients' tis-

TABLE I
Record of Transmission Experiments

Case No.	Inoculation of material*		Recovery of <i>B. granulosis</i> from patient	Inoculation of <i>B. granulosis</i> into monkeys	Recovery of <i>B. granulosis</i> from animals inoculated with human material
	Subconjunctival	Swab			
1	+++	+-	+	+	+
2	+-		-	Not recovered	-
3	+-		-	" "	+
4	++		+	+	No experiment
5	--		+	+	" "
6	--		-	Not recovered	" "
7	++		+	+	+
8	--		-	Not recovered	No experiment
9		+++	No experiment	No experiment	-

* Plus and minus signs in the first two columns represent for each sign the reaction of an individual animal.

sues yielded successful transfers to monkeys, whereas by the latter procedure, the swabbing of two patients' secretions induced granular conjunctivitis in animals.

The Experimental Disease.—The clinical appearance of the infected monkeys' conjunctivae was identical with that in the experimental disease induced by cultures of *Bacterium granulosis*. As described by Noguchi (3), the reaction consisted of a slowly progressing granular conjunctivitis, occurring first in the inoculated conjunctiva, and later spreading to the uninoculated membrane of both eyes. The outstanding features of the affection were congestion, edema, and thickening of the conjunctiva which was studded with numerous follicles,

covering the tarsal plates as well as the retrotarsal membrane. In the lower conjunctiva, similar but less marked changes occurred. In general, the disease in monkeys closely resembled the early stages of trachoma in man. In another article (4) we have alluded to the similarity of the clinical appearance of the experimental disease to Type I and IIa (MacCallan's (5) classification) of human trachoma, and we have shown that by the superimposition of secondary infections with ordinary bacteria, the condition in the monkeys can be changed so as to resemble the florid stage of human trachoma. Indeed, the monkeys employed for the latter experiments were selected from among those described in this paper.

The microscopic changes in the conjunctiva removed from the experimental animals resembled those found by Noguchi (3) and ourselves in tissues inoculated with cultures of *Bacterium granulosis*. Moreover, the histopathological lesions were similar to those of human trachoma. They consisted chiefly of numerous large, typical lymphoid follicles, scattered monocyctic infiltration, and thinning out or complete denudation of the epithelial layer. In addition, there was sparse scar tissue formation, especially in the subepithelial tissue and in a narrow zone around some follicles.

Recovery of Bacterium granulosis from Human and Monkey Lesions.—

The tissue obtained from one patient (Case 9) was insufficient in amount for cultivation tests, hence cultures were made with material from only eight of the patients. The material obtained from four (Cases 1, 4, 5, and 7) yielded growths of *Bacterium granulosis*. In addition, the microorganism was recovered from two other patients whose tissues were not employed for transmission experiments. All of the cultures obtained were shown to be pathogenic for monkeys: in each instance characteristic granular conjunctivitis was induced. In view of the fact that the patients had advanced trachoma with a considerable degree of secondary infection by ordinary bacteria and that the disease had been of long standing, the numerical results of cultivation are what one might expect.

Cultures of *Bacterium granulosis* were also obtained from the conjunctivae of the following monkeys which were infected with the tissues or secretions of the patients: Monkeys A and C, Case 1; Monkey F, inoculated with tissue from Case 3, and Monkey N, with that from

Case 7. Table I summarizes the results of the transmission experiments.

We have shown, therefore, that affected tissue from human trachoma, as it exists in white patients in New York City, induces in *Macacus rhesus* monkeys characteristic granular conjunctivitis. We have also found that *Bacterium granulosis* can be isolated from the same trachomatous tissues used for successful inoculation of monkeys and from animals thus infected.

These positive results with New York cases of trachoma, when taken with those with white patients in Denver and Chicago, show clearly that *Bacterium granulosis* is not peculiar to the trachoma of American Indians in Arizona. In this connection, the isolation of the same organism from natives of Russia, Italy, and Tunisia should also be stressed (6).

Transmission Experiments with Monkeys' Conjunctival Tissue and with Cultures of Bacterium granulosis

In planning the following experiments on transmission of the experimental granular conjunctivitis induced by inoculation either of human trachomatous tissues or of cultures of *Bacterium granulosis*, an effort was made to imitate the way in which the incitant of trachoma might be implanted on the conjunctiva of man, namely, by contact or by rubbing the eyelids (7). The first experiment concerned the conveyance of infection by simple contact.

Conveyance of Infection by Contact.—The source of infection in this test consisted of two monkeys (A1 and B1). Monkey A1 had been originally inoculated subconjunctivally with *Bacterium granulosis* (Albuquerque strain No. 1), isolated by Noguchi (3) in 1926, on Feb. 10, 1928. Monkey B1 had also been inoculated with the same strain derived from a monkey passage (1928), on May 17, 1929. In both cases characteristic progressive chronic granular conjunctivitis resulted and on Nov. 8, 1929, when the contact test was begun, the conjunctival lesions were well marked. Two monkeys (C1 and D1), having smooth conjunctivae, were then placed in the same cage with Monkeys A1 and B1. They were examined on Nov. 26, 1929 (18 days later) and both showed definite signs of characteristic granular conjunctivitis in both eyes. In the case of Monkey C1, the congestion, edema, and follicles persisted for 198 days. Thereafter the conjunctiva healed. The condition in Monkey D1 is still present without any sign of amelioration (at the time of writing, the disease has endured for about 15 months). On Feb. 13, 1930, 79 days after the disease was established, a culture of *Bacterium granulosis* was recovered from the conjunctiva of this monkey.

The experiment was repeated. The sources of infection were two monkeys, originally inoculated successfully by the use of another strain of *Bacterium granulosis*. Two additional monkeys with smooth conjunctivae were caged together with the affected monkeys, and after 14 days' contact, the exposed animals revealed, in both eyes, characteristic granular conjunctivitis which is still persisting, now 10 months later. One of the monkeys infected by contact served for cultivation tests and *Bacterium granulosis* was recovered from its conjunctiva when the lesions were well advanced.

That contact can play a rôle in the extension of lesions has already been shown by Noguchi (3), who pointed out that lesions ultimately appear on the uninoculated conjunctiva of the infected monkey. The foregoing experiments show that infection can be secured merely by caging together uninoculated and inoculated animals. Similar results of infection by contact were obtained by Finnoff and Thygeson (1). *Transfer of Infection by Instillation of Cultures or Tissues.*—In this test an attempt was made to stimulate in monkeys other conditions which might initiate infection in man. Cultures were instilled and the eyelids rubbed.

Suspensions of three Arizona strains (2) of *Bacterium granulosis* were pooled and instilled into the conjunctival sac of each of two monkeys having smooth conjunctivae. The suspensions were dropped into the sac on each of 7 consecutive days, and after about 5 days' rest, two additional, similar series of daily instillations for 8 days were given. In all, 23 doses were administered. The monkeys remained unaffected.

In contrast with this control experiment, demonstrating the innocuousness of merely instilling suspensions of cultures, is the following test. Two monkeys, both with smooth conjunctivae, received in their conjunctival sacs about 5 drops of a suspension of the same cultures used in the foregoing experiment. After each of eight daily instillations, the eyelids were gently massaged for about a minute. 5 days after the last instillation and massage of the eyelids, both monkeys revealed characteristic granular conjunctivitis which persisted until their death from tuberculosis 6 weeks later. They had been kept in the same cage.⁴

In contrast, again, with the control experiment is the following: Into the conjunctival sac of two macaques having smooth conjunctivae was instilled a suspen-

⁴ We have not been able to find any effect of tuberculosis on experimental trachoma or vice versa. As many monkeys die of tuberculosis among isolated normal stock animals as amongst trachomatous monkeys. The longest duration of experimental trachoma without supervening tuberculosis is, in our experience, 4 years.

sion of affected monkey tissues. The suspension was prepared by scraping the conjunctival tissue from six monkeys showing granular conjunctivitis which had been induced by inoculation of *granulosis* cultures, and suspending the tissue particles, or scrapings, in saline solution. Seven daily instillations were given; and 24 days later, both animals revealed definite, progressive, granular conjunctivitis which, at the present time, 8 months after inoculation, still persists.

The repeated simple instillation of cultures of *Bacterium granulosis* into the conjunctival sac of normal monkeys did not appear to infect the animals. On the other hand, the daily instillation of cultures followed by gentle rubbing of the eyelids after each instillation induced characteristic granular conjunctivitis. In addition, suspensions of conjunctival tissue freshly removed from monkeys having *granulosis* conjunctivitis, instilled repeatedly into the conjunctival sac of normal animals, gave rise to the characteristic experimental disease. The findings made it seem likely that some injury, even so slight a one as that produced by rubbing the eyelids, is a requirement for infection by the microorganism.

Conveyance of Infection by Swabbing Monkey Secretions and Cultures.—The transfer of the experimental disease of monkeys to normal animals was now attempted by swabbing secretions on the conjunctivae, thus imitating the mode of conveyance of infection from man to monkey, already described. And following this, another test was made to determine if swabbing with cultures of *Bacterium granulosis* would induce the infection.

Test with Secretions.—The source of the secretions was a *Macacus rhesus* monkey inoculated subconjunctivally in Feb., 1928, with tissue from another monkey infected with Albuquerque No. 1 strain of *Bacterium granulosis*. The conjunctival lesions were advanced and had endured for 22 months when the secretions were taken for the test. The material was collected on cotton swabs and transferred directly, by rubbing, to the smooth upper and lower conjunctivae of both eyes of two *rhesus* monkeys (A2 and B2). After six such swabbings, over a period of 8 days, the animals had developed characteristic granular conjunctivitis in both eyes. In Monkey A2 the lesions were progressive and endured for 2½ months, after which the animal died from tuberculosis. In Monkey B2 the condition still persists, after 1 year. A culture of *Bacterium granulosis* was recovered from the animal 10½ months after the disease first appeared.

Test with Cultures.—The cultures employed were the three Arizona strains (2) previously used in the instillation experiment described above. Saline suspensions of pooled growths were swabbed in the manner already described, on the clear

conjunctivae of two monkeys. Monkey A3 was swabbed 30 times in three series of eight and one of six daily dosages, with a rest interval of 8 days between series. Monkey B3 received only the first series of eight daily swabbings. Both monkeys developed characteristic granular conjunctivitis 4 days after the last dosage. In both monkeys, the lesions are still present, now after 1 year. From Monkey A3 a culture of *Bacterium granulosis* was recovered in the 7th month of the disease. No cultivation test was made on Monkey B3. Saline suspensions of uninoculated leptospira medium, such as were employed for growth of the cultures, proved innocuous when swabbed upon monkey conjunctivae in precisely the same way.

It is plain that experimental trachomatous conjunctivitis can be produced in *Macacus rhesus* monkeys by repeatedly swabbing the conjunctivae with secretions from animals having *granulosis* conjunctivitis in an advanced stage, or with cultures of *Bacterium granulosis*.

SUMMARY AND CONCLUSIONS

1. Conjunctival tissue derived from alien and native American white persons in New York City, having trachoma in an advanced stage, has been used successfully to induce in *Macacus rhesus* monkeys characteristic granular conjunctivitis. The transfer of infection was effected either by a single subconjunctival injection, or by repeated swabbing with conjunctival secretions.
2. Pathogenic strains of *Bacterium granulosis* have been recovered from the trachomatous tissues of six out of eleven patients. In addition, the organisms have been isolated from the monkeys infected with human material.
3. Repeated swabbing with secretions obtained from monkeys having experimental trachoma has given rise to characteristic granular conjunctivitis in normal animals. In addition, repeated instillations of suspensions of conjunctival tissue fragments derived from affected monkeys have led to characteristic infection of the conjunctivae of normal monkeys.
4. Contact infection occurs in monkeys, as it has long been known to occur in human beings; animals with smooth conjunctivae developing the experimental disease when merely caged with infected monkeys.
5. Repeated instillation of cultures followed by rubbing the eyelids will lead to the disease in monkeys, a method of transfer which indicates one manner in which the affection may be transmitted from

man to man. Yet another manner of producing the experimental condition is by repeated swabbing with cultures of *Bacterium granulosis*. Noguchi has already reported the successful outcome of the sub-conjunctival inoculation of cultures and the spread of the disease from an infected conjunctiva to the other eye of the same animal.

6. Tissues derived from cases of human trachoma or from monkeys having the experimental disease induce, on conjunctival inoculation of *Macacus rhesus* monkeys, the same clinical and pathological effects as do cultures of *Bacterium granulosis*. The conjunctival lesions closely resemble, in clinical appearance and in microscopic changes, those of the follicular stages of trachoma in man.

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THE PATHOLOGY OF EXPERIMENTAL DERMAL PNEUMOCOCCUS INFECTION IN THE RABBIT

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PLATES 2 AND 3

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The development and progression of the morphological changes in the human pneumonic lung are still incompletely understood, a fact partly explainable by the circumstance that pneumonic lesions, lobar in distribution, can be produced in animals only with difficulty. In previous papers (1, 2) one of us has described an experimental disease brought about by infecting rabbits intradermally with pneumococci (Type I). Although in this infection the focus is in the skin, there exist certain clinical and immunological similarities with lobar pneumonia in man; moreover, the visible location of the principal lesion is a definite aid to experimental studies. Observations made in the course of other experiments of this nature suggested that a study of the morphological alterations, both gross and microscopical, might lead to a better understanding of the fundamental phenomena of pneumococcic infection irrespective of the site of the lesion.

It may be recalled from an earlier report (1) that if a normal rabbit is given an intradermal inoculation of virulent pneumococci on the flank a local infection develops in the course of several hours. The lesion soon begins to spread ventrally and within 24 hours occupies a large area extending from the site of inoculation across the ventral midline. The infection is characterized particularly by the production of massive edema.

The course of the development of the lesion, as previously reported (1), may be summarized as follows:

After the intradermal injection of 0.2 cc. of an 18 hour blood broth culture there is at first a latent period of some 2 hours. Near the site of inoculation there then appear signs of early inflammation; these are characterized at first by conges-

tion of vessels and capillaries; the sharp vascular outlines are then lost and an area 1 to 2 cm. in diameter becomes slightly edematous and shows a trace of orange-red color. As more edema develops the accumulation of fluid is most pronounced at the ventral border. The lesion spreads ventrally at a rate of 2 to 3 cm. an hour, the heightened color being apparently preceded by the movement of fluid. At 10 to 12 hours the edematous band has usually extended to the ventral midline. More and more serous material accumulates in this region until the entire area is tense and swollen. Within 18 to 24 hours a certain degree of induration is present and this increases gradually until at 48 hours the involved tissue is firm and relatively non-elastic.

At 24 hours the lesion usually occupies a strip 2 to 3 cm. wide on the flank increasing to a width of 4 to 6 cm. at the ventral midline. Beginning at 24 to 30 hours there is a marked contraction of the involved tissue, and large irregular folds develop over the more ventral section. The color of the lesion varies from a pale to a moderately bright orange-red. In the infections which appear to be the more severe and fatal the involved skin often takes on a hemorrhagic character frequently evidenced by scattered ecchymoses or by widespread hemorrhage. Some frank necrosis with secondary infection may take place, the incidence varying with the amount of trauma to which the tissue has been subjected.

Methods

In order to study the histological development of this dermal lesion, a series of normal animals were injected intradermally with 0.2 cc. of an 18 hour blood broth culture of *Pneumococcus* Type I. The site of injection in each instance was at a point on the animal's flank approximately 8 cm. dorsal to the ventral midline. The development of the lesions and the concomitant temperature reactions were then carefully followed.

Except in one instance the animals were killed at various intervals after infection by inducing air embolism. The entire skin lesion, including the underlying muscle and peritoneum, was removed, lashed to cardboard to prevent distortion and by a longitudinal incision divided into two equal pieces. One was fixed in Zenker-formol and the other in Zenker's fluid to which 5 per cent of glacial acetic acid had been added. Multiple blocks, suitable for study of the entire extent of the lesion were embedded in paraffin, sectioned, and stained with hematoxylin-eosin, eosin-methylene blue, and Mallory's phosphotungstic acid hematoxylin. Stains for bacteria were made according to the method of Brown (3).

HISTOLOGICAL OBSERVATIONS

The earliest lesion to be examined was removed 2 hours after infective inoculation and before any elevation of the animal's temperature had taken place.

The only external evidence of disease was a soft, pale area of very slight swelling about the point of inoculation. Histological examination shows a marked accumu-

lation of edema fluid in the corium and in the loose adipose and connective tissue between corium and muscle. A moderate number of polymorphonuclear leukocytes are present although fibrin is not seen nor are erythrocytes in evidence in any considerable number. The muscle layer and corium show scattered infiltration with leukocytes. Blood vessels, particularly the smaller ones, are congested, dilated, and appear more numerous than in the normal skin. The lymphatics also appear larger and more numerous but contain few leukocytes and no fibrin. Study of the borders of the lesion indicates clearly that extension of the edema fluid takes place in advance of infiltration with inflammatory cells. No evidence of tissue necrosis can be made out. Nearer the center of the lesion, presumably in relation to the site of inoculation, a marked accumulation of mononuclear wandering cells has taken place in and around the adventitia of the blood vessels. A photograph of the lesion at this stage is reproduced in Fig. 1. This shows the early histological development with edema distending the corium and subcorial space. Fig. 2 shows the uninvolved tissue just peripheral to the lesion.

The progression of the histological alterations was next studied in a lesion taken at 5 hours.

This animal's temperature had risen to 104°F., an elevation of a little over a degree from the normal. The involvement at this time measured 2.5 by 3.5 cm. the latter being the dorsoventrad dimension. This area was definitely edematous, a feature most prominent at the ventral border. The color except for the most ventral portion was a faint orange-red and the consistency very soft.

At this time the histological picture generally parallels that described at 2 hours, but all features are more marked. The loose connective tissue fibers of the corium and panniculus adiposus have been widely separated from each other by the edema fluid, and this has resulted in a structure which, on superficial examination, appears not unlike the areolar structure of pulmonary parenchyma. The relatively thin tissue layer has thus been converted into the outstanding anatomical structure. The cellular infiltration has extended into the deeper layers of the corium but as yet is not a marked feature. The dilatation of the lymphatics and the increase in size and number of functioning vessels present a picture similar to that seen in the earlier specimens. Despite the size and rapid progression of the lesion, the response in the form of cellular exudate is only moderate in degree. Necrosis of tissue cannot be detected, nor can any deposition of fibrin be observed. Although dilated lymphatics and engorged blood vessels form a prominent feature at this stage, even more so than in the earlier lesion, no evidence of thrombosis can be found.

The next tissue studied was removed from an animal 8 hours after infective inoculation.

The rabbit's temperature had risen to 105°F. The lesion occupied an area 2.5 by 8.5 cm. extending to and just beyond the ventral midline. The entire zone was soft and edematous, a change most pronounced at the more ventral projection. Near the point of original inoculation the color was a very bright orange-red but this shaded off gradually until the ventral third of the area was of the same color as the surrounding tissue. At this time sections show that there had been a distinct shift in the predominant cell type of the inflammatory reaction. The perivascular grouping of mononuclear phagocytic cells is a striking feature, many cells of this type having also taken part in the general cellular response. Edema is still the main feature of the lesion and has completely separated corium from muscle. Infiltration, both of polymorphonuclear and mononuclear cells, is steadily increasing in degree and extends, at this time, well into both corium and muscle layers. Still no hemorrhage or necrosis is present. The degree of lymphatic dilatation does not appear to be altered but more small blood vessels, greatly engorged, are appearing in the corium.

The further progress of the pathological processes was studied in a lesion removed at 28 hours.

The animal's temperature had been over 104°F. for approximately 20 hours, with a maximum high level of 105.5°F. The lesion at this time occupied a strip 3 cm. wide extending downward from the point of inoculation to the ventral midline where the involvement measured 8 cm. in diameter. The entire area was greatly swollen, an alteration accentuated by the formation of huge folds caused by the contraction of the skin. The lesion was rather firm, especially in the area near the site of inoculation. The color varied from a pale to a bright orange-red. No macroscopic necrosis or hemorrhage was apparent. Microscopically, the sections show at the site of maximum involvement, near the juncture of upper and middle third of the lesion, the earliest evidence of death of the cells forming the exudate. Here groups of acidophilic mononuclear and polymorphonuclear leukocytes are seen. Peripheral to this reaction, the picture previously described of corial and subcorial edema associated with mild inflammatory infiltration is encountered. Perivascular cell infiltration is still marked and the large number of newly formed or newly functioning capillaries is most striking. The lower edge of the lesion, on the other hand, as the most recently involved, presents changes not unlike those seen in the preparations previously described. Here, the edema, both in the subcorial space and in the inferior layers of the corium, is by far the most prominent change. Inflammatory exudate is sparse and diffuse.

As an example of the fully developed lesion, tissue was studied 2½ days after infection.

This animal died under observation after a typical course of the disease associated with high temperature. The tissue was excised and fixed immediately

after death. The lesion occupied an area of much the same size as that last described. The edema was massive, firm, and circumscribed. A bright orange-red color, most pronounced in the early phases, was succeeded at 45 hours by ecchymoses which rapidly progressed to hemorrhagic necrosis. At this time a radical alteration appears in the histological picture at the oldest part of the lesion, situated well toward the dorsal end. Here, in addition to the edema previously described, fairly dense infiltration composed of mononuclear and polymorphonuclear leukocytes is found in the lower layers of corium and the strata of the upper muscle layers. Groups of necrotic inflammatory cells are occasionally encountered although necrosis of parenchyma is not apparent. Diffuse hemorrhagic infiltration is striking in certain areas. Peripheral to this well established lesion, there are regions presenting rather less activity, where the edema is most prominent and the cellular infiltration not marked. A large number of new or newly functioning capillaries and even larger vessels have appeared, many surrounded by a collar of mononuclear cells. Some proliferation of fibroblasts has taken place in the lower corium. The epithelial surface presents a number of small areas where the cells have undergone a degenerative change overlying a base of coagulated exudate containing roughly parallel rows of polymorphonuclear and mononuclear leukocytes. Subepithelial lymphatics are greatly distended. Inflammatory infiltration extends well up to the epithelium and down to the second layer of muscle. A certain number of muscle fibers appear necrotic. Thrombosis of both large and small veins is well marked in this preparation, a condition presumably accountable for the marked hemorrhage present.

The last example studied was that of an advanced lesion, removed at 99 hours after infection.

This presented essentially the same gross pathological picture as that just described, except that the hemorrhagic condition was never so prominent. Microscopically, this lesion shows a picture unlike that seen at any other period studied. The process is one of repair and of reaction to foreign material represented by groups of cellular débris. Much edema is still present and there is a massive infiltration with leukocytes, predominantly mononuclear in type. Many new vessels and young active fibroblasts are seen, a change particularly marked around the pockets of necrotic material. Thrombosis is a marked feature. The extensive edema and scattered cellular infiltration, so marked in the earlier slides, is not apparent in this later preparation. Figs. 3 and 4 show low and high magnifications of a section of a mature lesion.

These examples show the course of development and maturation of the lesion. Studies on the histological changes occurring during resolution are now in progress and will be presented in connection with certain data bearing on the recovery process.

DISCUSSION

In order to relate these observations to the problem of the early morphological alterations in lobar pneumonia, it is first necessary to consider in some detail the changes which take place in the human lung. Briefly, these changes have been described as engorgement, red hepatization, gray hepatization, and resolution.

The first stage, that of engorgement, is not commonly seen at autopsy and the descriptions for the most part have been derived from study of tissue contiguous to a pneumonic process in the direction in which the pneumonic process is presumed to be spreading. Here engorged vessels may often be found. More commonly, however, the only evidence of progression is the accumulation, in the alveoli, of edema fluid containing a few polymorphonuclear leukocytes. Blake and Cecil (4), as well as Winternitz and his coworkers (5) have called attention to the dilated lymphatics in experimental pneumococcus pneumonias in animals. Permar (6), on the other hand, studied the lesion of experimental pneumonias sooner after infection than was done by the workers just mentioned and pointed out that the most striking change was the presence of intraalveolar fluid in the absence of lymphatic involvement. Loeschcke (7), in a detailed study of the histological alterations in the human pneumonic lung, has called attention to the prominence of edema formation in the early and advancing lesions.

Comparing these changes, considered to be the earliest manifest in the pneumonic lung, with the alterations observed in the infected skin of the rabbit a remarkable similarity is immediately evident. The accumulation of a relatively cell-free exudate and the presence of dilated lymphatics, dilated and engorged capillaries, and an intact supporting structure are all found in the dermal infection. The progression of the infection by the production of edema fluid, presumably containing the infecting bacteria, would suggest strongly that a similar mechanism may obtain in the pneumonic lung.

The stage of red hepatization is only rarely encountered in lobar pneumonia. It is usually described, however, as being characterized by a deep red color, presumably due to dilated and engorged capillaries of the alveolar wall. The exudate stains well and contains a considerable number of polymorphonuclear leukocytes and a certain amount of fibrin, although the latter is a variable factor. The interstitial tissue is still intact and the lymphatics are dilated, containing a certain number of inflammatory cells.

Turning to the lesion observed in the rabbit skin, the same general pathological alterations are found. Vascular congestion is a marked

feature and the inflamed area appears red on gross inspection. The exudate near the site of inoculation now contains a larger number of inflammatory cells, mostly polymorphonuclear leukocytes, as well as a few mononuclears. The number of erythrocytes is extremely variable. Fibrin may be present but it is not a striking feature. The lymphatics are markedly dilated and may contain a moderate number of leukocytes. As in the lung no evidence of damage to the supporting structure is encountered. At the periphery of the progressing lesion, the changes are identical with those of the initial reaction present throughout when the tissue is examined a few hours after inoculation. Ex-lung, where, at the periphery of the consolidated tissue, areas may be found in which the alveoli contain edema fluid with a few cells and the vessels are greatly engorged, a picture presumed to be widespread early in the disease. As in the lung no necrosis is to be found, so also, the interstitial tissue of the rabbit skin is quite intact.

The third stage of lobar pneumonia, that of gray hepatization, is the one most frequently encountered at autopsy. Here, the fibrin of the exudate is commonly most dense and shows a tendency to shrink away from the alveolar walls. The cellular exudate, composed for the most part of polymorphonuclear and mononuclear leukocytes, stains poorly and is presumed to be largely necrotic. The small vessels in the walls of the alveoli are inconspicuous and may contain no blood whatever though this can hardly be stated to be the rule. A number of mononuclear phagocytic cells have appeared by this time, and many contain droplets of fat or fine granules of hemosiderin.

The comparison with the more advanced lesion of the rabbit dermal infection is a striking one. Here, corresponding to the pneumonic lung, one finds that the oldest exudate is necrotic and that mononuclear phagocytes are engaged in carrying away various broken-down products. Capillary engorgement is by no means so marked and although many new vessels have appeared they are in association with granulation tissue, a change easily understood when one considers that in the skin the exudate has no ready route of exit. The removal of the exudate must take place more slowly and a certain amount of organization is bound to occur. Necrosis of dermal tissue is, however, not a feature and in this respect the analogy with the pneumonic lung still holds. This analogy is also true in the case of the fibrin which is

much more dense in both of the lesions at this stage. One feature deserves especial attention and that is the occurrence of hemorrhage. In the dermal lesion this is fairly striking in certain of the more advanced stages but it has not been commonly described in discussion of the pathology of lobar pneumonia. Loeschcke (7) has recently called attention to the occurrence of intraalveolar hemorrhage in the later stages of lobar pneumonia. This author explains his finding by the shrinkage of the fibrin plugs away from the alveolar walls, allowing a decrease of pressure on the alveolar side of the membrane and consequent rupture of the weakened vascular wall with resultant outpouring of red blood cells into the alveoli. It is undoubtedly true that hemorrhagic areas are frequently observed in the pneumonic lung at autopsy and a detailed microscopic study of a limited amount of material available to us has revealed a surprising amount of intra-alveolar hemorrhage in areas of the lung known from clinical data to represent the older pathological processes. In such preparations thrombosis of the finer vessels appears to give rise to this phenomenon and here again the same condition is found in the skin lesion.

In recapitulation, it may be said that the predominant change in the rabbit skin, infected with pneumococci, appears to be the production of an extraordinary volume of edema fluid. This fluid, carrying pneumococci, seems to spread rapidly, filling the interstices of the connective tissue framework. The direction which the fluid takes is influenced principally by gravity. Following the production of fluid there appears a cellular exudate, predominantly polymorphonuclear but in the later stages containing also many mononuclear cells. As the age of the lesion increases more fibrin is seen and eventually the oldest exudate becomes necrotic, although at the border the relatively pure edematous lesion is still present. Hemorrhage may be a late manifestation. Since changes of the same general nature may be observed in the pneumonic lung, it is of some interest to consider the likelihood that the pulmonary disease may extend by a similar mechanism, that is to say, by the production of an infectious edema fluid near the hilus with secondary spread of this fluid by force of gravity and by the churning action of the moving alveolar walls.

The chief immediate significance of these findings is that they establish the fundamental similarity of the pathological processes of the

rabbit dermal disease and those of human lobar pneumonia. This similarity has importance for work to be reported later on factors which affect the development, progression, and localization of the rabbit lesion, as well as in relation to the method that has been suggested for the comparison of the curative properties of antipneumococcic sera by use of this experimental infection (8).

SUMMARY

1. The pathology of the experimental dermal pneumococcic infection in the rabbit is described in detail and the findings are compared with the histological alterations seen in the human pneumonic lung. There would appear to be a basic similarity of the lesions in both tissues.

2. A copious production of edema fluid is the outstanding characteristic of the early lesion. It occurs prior to any significant cellular change. In the spreading lesion an infiltration of the tissues with fluid precedes any other sign of reaction between tissue and microorganism. It seems likely that the advancing fluid carries with it the infecting organisms and inoculates all tissues which it reaches. The resulting infection seems not to take place by an active invasion of microorganisms but by a progressive inoculation from an infected fluid.

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EXPLANATION OF PLATES

PLATE 2

FIG. 1. Photomicrograph of a section of a lesion from the skin of a rabbit 2 hours after infection with *Pneumococcus*, showing marked edema of corium and subcorial space associated with slight cellular infiltration. $\times 65$. Phosphotungstic acid hematoxylin.

FIG. 2. Same section, showing relatively unaffected tissue beyond the periphery of the lesion depicted in Fig. 1. $\times 65$. Phosphotungstic acid hematoxylin.

PLATE 3

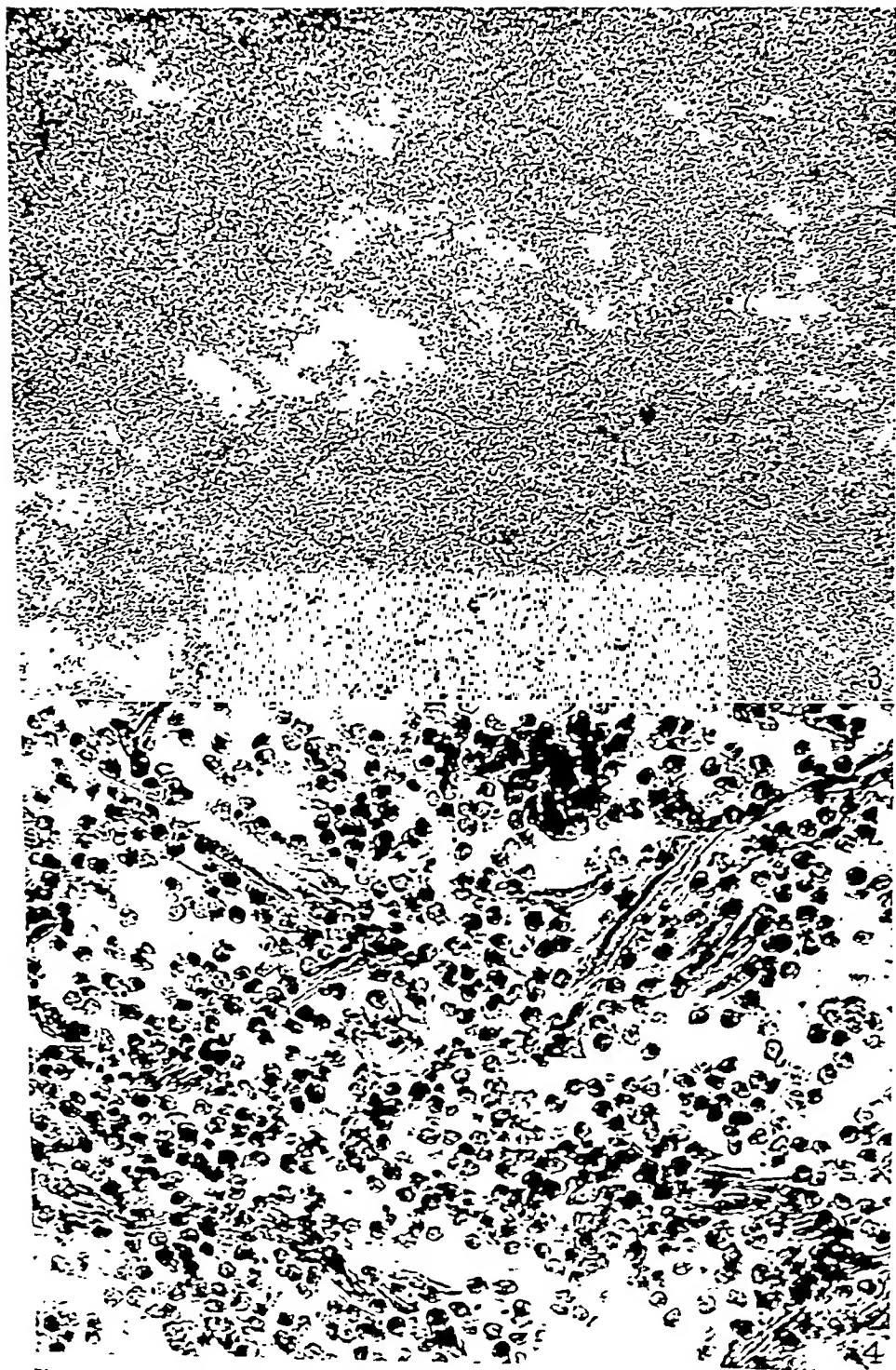
FIG. 3. Low power photomicrograph of corium showing the intense inflammatory infiltration in the mature lesion. $\times 65$. Eosin-methylene blue.

FIG. 4. Same section, with higher magnification, indicating the marked edema, the character of the cellular infiltration, and the separation of connective tissue fibrils. $\times 550$. Eosin-methylene blue.



Photographed by Louis Schmidt

(Rhoads and Goodner: Dermal pneumococcus infection)



Photographed by Louis Schmidt

(Rhoads and Goheen: Dermal pneumococcus infection)

DECOMPOSITION OF THE CAPSULAR POLYSACCHARIDE OF PNEUMOCOCCUS TYPE III BY A BACTERIAL ENZYME

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It is now generally recognized that one of the important differences between the two variants of *Pneumococcus*, the so called R and S forms, is the presence around the latter of a capsule which has been shown to contain a complex polysaccharide; the type specificity of *Pneumococcus* and the virulence of the S cells are associated with the presence of this capsular polysaccharide which is referred to as the soluble specific substance; the chemical structure of the capsular polysaccharides has been shown to vary from one type of *Pneumococcus* to another (1); in fact, they are as chemically distinct one from the other as they are serologically specific.

The fact that the capsular polysaccharides of *Pneumococcus*—and not some impurities carried along with them—are themselves the substances responsible for type specificity, has been shown by the disappearance of their reactivity in the specific antisera after they had been subjected to acid hydrolysis. However, this treatment is a fairly drastic one and may have affected at the same time the hypothetical impurities. It was thought that such an objection would be removed if the polysaccharide could be split by the milder action of an enzyme.

It was interesting also to determine whether the addition of such an enzyme to a medium seeded with the encapsulated pneumococci would effect a dissolution of the capsule. From this point of view the observations of Toennissen (2) are of special interest. He found that when *Bacillus vulgatus* was seeded together with encapsulated Friedländer bacilli, the latter organisms grew deprived of their capsule. Finally, we had in view experiments to determine whether the injection into the animal body of an enzyme capable of decomposing the cap-

sular polysaccharide would in any way alter the course of experimental infection induced by *Pneumococcus* of the same type as that from which the specific substance had been derived.

A systematic search for specific enzymes of this order has been carried on in this laboratory for several years. A number of enzymes from animal and plant sources, known to be active in the hydrolysis of simpler carbohydrates, were tested, but none of them were found capable of attacking the polysaccharides of pneumococcus origin. In addition, cultures of various molds, yeasts, soil actinomycetes, and bacteria, many of which were known to decompose cellulose and other complex carbohydrates, were tested without success.

Recently, however, a microorganism has been isolated, and an enzyme extracted from it, both of which are capable of decomposing the capsular polysaccharide of Type III *Pneumococcus* (3). The object of this first paper is to describe the technique of isolation, the morphological, cultural, and biological characteristics of the organism; and to define the nature and mode of action of the enzyme derived from the bacterial cells.

EXPERIMENTAL

1. Inoculation Material.—As mentioned previously, many organisms known to decompose actively various polysaccharides were tried and were found unable to decompose the capsular polysaccharides of *Pneumococcus*. Since these specific substances have many of the properties of hemicelluloses, an attempt was made to search in a natural environment for organisms possessing the capacity to split complex substances of this nature. It was thought that those locations where large amounts of organic materials,—especially belonging to the group of “hemicelluloses,”—accumulate and undergo decomposition, were the most likely to harbor the desired organisms. Among the materials tried were leaf mold, composts (of corn cob, rye straw, sphagnum, oak leaves, etc.), farm manure, soils rich in organic matter (different peat soils and soils heavily manured).¹ From a

¹ Many of these materials and especially the peat samples from the cranberry bogs of New Jersey were supplied by Dr. S. A. Waksman and Dr. R. L. Starkey of the Department of Microbiology of the New Jersey Experiment Station to whom we extend our heartiest thanks for their courtesy and cooperation.

sample of soil from the cranberry bogs of New Jersey a microorganism has been isolated which is capable of decomposing the capsular polysaccharide of *Pneumococcus* Type III.

2. *Medium*.—The mineral medium used was based on one previously described for the isolation of cellulose-decomposing bacteria (4).

Ammonium sulfate (1 gm. per liter) was used as a source of nitrogen, dibasic potassium phosphate (2 gm. per liter) as buffering agent and source of phosphate, tap water supplied traces of the other mineral elements. The reaction of the medium was adjusted to the proper pH with HCl and NaOH. To this mineral solution, the capsular polysaccharide of Type III *Pneumococcus* was added in final concentrations varying from 0.001 to 0.2 per cent. This substance was the only source of organic carbon in the medium.

The soluble specific substance used in these experiments was prepared from a strain of Type III *Pneumococcus* by the method previously described (1, 5). This nitrogen-free preparation yields on hydrolysis a mixture of aldobionic acid and glucose. However, the molecule seems to be built up exclusively of aldobionic acid groups, the glucose appearing only as a secondary product of hydrolysis (6).

The use of this simple and specific medium was dictated by the following considerations.

(a) It was possible that the materials used for inoculation contained organisms *potentially* capable of decomposing the specific substance but for which other nutrients would act as more readily available sources of energy. Such organisms would attack the specific substance when deprived of any other food but leave it untouched in a complex medium. In fact, the production of an enzyme is often the result of what has been termed a "starvation" phenomenon. For example, Brown and Morris (7) found that the secretion of diastase by germinating barley seeds is inhibited by the presence in the medium of sugars which can be utilized directly by the growing plantlet. In a review on bacterial enzymes, Waksman has cited many similar examples which occur in the microbial world (8). Wortman (9) for instance, found that a certain bacterium had the power of excreting a starch-dissolving enzyme when starch was the only available food, but that no secretion of enzyme occurred if sugar or tartaric acid was offered to the organism along with the starch.

(b) The material used for inoculation was of course a mixture of a

great variety of microbial species. A medium containing the specific substance as sole source of carbon rendered conditions favorable only for these organisms capable of utilizing the specific substance itself or the products of its decomposition. This procedure afforded a means of rapidly eliminating a large number of irrelevant species.

(c) The ultimate object of the work was the preparation of an enzyme specifically directed against the capsular polysaccharide. For study of the action of the enzyme on the growth of *Pneumococcus*, and on the course of pneumococcus infection in experimental animals, it was especially desirable to have a preparation as poor as possible in proteolytic activity. From this point of view, it was expedient to use a mineral source of nitrogen instead of peptone or protein.

3. *Technique of Isolation*.—In order to eliminate as many as possible of the organisms which although unable to attack the specific substance may grow on the products of its decomposition or on the bacterial bodies, transfers were made as soon as growth could be detected in the cultures, in the hope that the first organisms to develop would be the ones attacking the specific substance and that in young cultures they would outnumber the others which could then be eliminated by diluting the inoculum.

4. *Serological Method for Following the Disappearance of the Specific Substance*.—The presence or absence of the specific substance in a culture was tested by the precipitin reaction:

0.5 cc. of fluid to be tested was added to 0.2 cc. of Type III antiserum² and the mixture brought to a volume of 1 cc. by the addition of salt solution. Since the precipitation test gives a positive result with a concentration of specific substance as low as 1:5,000,000, the absence of a positive precipitin reaction was interpreted as evidence of complete decomposition of the specific substance.

The Organism

1. *Isolation*.—The presence in a sample of soil from the cranberry bogs of New Jersey, of an agent capable of decomposing the Type III specific substance, is demonstrated in the following experiment.

² The Type III antipneumococcus serum used in these experiments was furnished through the courtesy of Dr. A. B. Wadsworth, Director of the Division of Laboratories of New York State Department of Health.

Experiment 1. The Effect of Environmental Conditions (pH, Temperature, Aeration) on the Decomposition of the Capsular Polysaccharide by an Agent Present in Peat Soil.—Different lots of basic mineral medium containing 0.002 per cent of Type III specific substance were adjusted to pH 4.5, 5.0, 5.5, 6.2, 6.6, 7.0, 7.8, and 8.5. Each one of these media was divided into three small Erlenmeyer flasks (25 cc. per flask) and into three Noguchi tubes (10 cc. per tube). Each of the tubes and flasks was inoculated with about 0.5 gm. of peat soil (from the cranberry bogs of New Jersey). The Noguchi tubes were incubated under anaerobic conditions (Brown jar) and the Erlenmeyer flasks under aerobic conditions. In each instance one set was kept at room temperature (about 22°C.), a second set at 37.5°C., and a third set at 54°C.

The cultures, in mineral media at different hydrogen ion concentrations and incubated at different temperatures under aerobic and anaerobic conditions, were tested from time to time for the presence of the specific substance. The specific precipitin reaction became negative first in the aerobic flask at pH 7.8, after 24 days incubation at 37.5°C. Within the following 10 days, the test became negative also in all the aerobic cultures at pH 6.2, 6.6, 7.0, and 7.8 at room temperature and 37.5°C. The specific substance did not disappear in the other flasks nor in the anaerobic tubes, even after 2 months incubation.

These results pointed to the existence of an agent capable of decomposing or removing from solution the specific substance between pH 6.2 and 7.8, at room temperature and 37.5°C., under aerobic conditions. That it was a living agent was shown by the fact that it could be transferred in series.

Since we had in view the use of this agent in the animal body, it was advisable to incubate the cultures at 37.5°C. in a medium of approximately neutral reaction; these conditions approach those present in the animal body and had been found in Experiment 1 to be close to the optimum for the activity of the microorganism.

Experiment 2. Attempts to Increase the Activity of the Culture by Repeated Transfers in Specific Medium.—Test tubes containing 10 cc. of the synthetic medium adjusted to pH 7.5 were inoculated with material from the aerobic flask in which the specific substance had first disappeared in Experiment 1. The incubation was carried on at 37.5°C., aerobically.

In this first transfer, the specific substance disappeared in 10 days. The culture was carried in the same medium for several months, transfers being made as soon as the specific substance had been decomposed,

in the hope of increasing the activity of the culture. In fact, after repeated transfers for 6 months, decomposition of 0.002 per cent of specific substance could be obtained regularly in 24 hours provided a young culture was used.

Experiment 3. Attempts to Purify the Mixed Culture by the Dilution Method.—A tube of synthetic medium was inoculated with a loopful of an active culture. 8 hours later stained films showed a fairly abundant growth of many different kinds of bacteria. At this time transfers were made into a series of tubes containing fresh mineral medium, using the following inocula: 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0.000001 cc.

After 18 hours incubation at 37.5°C. stained preparations of these cultures showed that growth had taken place in the first four tubes of the series. Serial transfers were immediately made from the fourth tube (0.0001 cc. inoculum) into fresh medium, using seedings varying from 0.1 to 0.00001 cc. of culture. 11 hours later growth was recognized (by microscopic examination) in the tube which received 0.001 cc. inoculum and from this culture serial transfers were again made.

The technique of purification by the dilution method was continued for 10 days. Each transfer was kept long enough to make sure that the active culture was carried along, as demonstrated by decomposition of the specific substance in 1 to 5 days, the time depending upon the size of the inoculum. Microscopic examination of a young culture of the last of the serial transfers, revealed the presence of only three morphological types of bacilli, a very small Gram-negative rod, a short plump Gram-negative rod, and a large Gram-positive rod. The impure culture thus obtained was plated in the hope of separating these three organisms, but unfortunately one of them was a spreader which overgrew the two other species.

Experiment 4. The Use of Gentian Violet in the Isolation of the Specific Organism from Mixed Cultures.—An active culture was seeded into mineral medium to which had been added 0.001 per cent of gentian violet in the hope that the bacteriostatic action of the dye would inhibit the development of the Gram-positive organism (10). 4 days later, the specific substance could no longer be detected in the culture containing gentian violet and microscopic examination showed the presence of only the two Gram-negative organisms, and a few large spores, apparently belonging to the smaller bacillus.

In the hope that the spore-bearing bacillus was the active form in the mixture, the following experiment was carried out.

Experiment 5. Isolation in Pure Culture of the Specifically Active Spore-Forming Bacillus by Heating the Inoculum at 70°C.—0.2 cc. of the culture just described was inoculated into two tubes of mineral medium containing 0.002 per cent of specific substance; one tube was then heated at 70°C. and the other at boiling temperature for 15 minutes.

After 7 days incubation at 37.5°C. the specific substance had been decomposed in the tube heated at 70°C. but it remained unaffected in the tube which had been boiled. Transfers made from the culture heated at 70°C. grew and decomposed 0.002 per cent of specific substance in 2 days; stained films of this culture showed what appeared to be a pure culture of a small, Gram-negative, spore-bearing rod, exhibiting metachromatic granules.

Young cultures of this organism plated on nutrient agar gave a pure growth of colonies which will be described later, whereas older cultures in the spore stage (5 to 6 days old) failed to grow on agar. Transfers were made from one of these colonies, and the Gram-negative bacillus in pure culture was carried for fifteen generations on blood agar from colony to colony. A colony from the fifteenth transfer was then inoculated into the mineral medium containing 0.002 per cent of Type III capsular polysaccharide. The latter was decomposed in 3 days, the culture showing again a pure growth of Gram-negative rods.

These experiments established the fact that the spore-bearing Gram-negative rod, and not some other agent carried along with it, is responsible for the decomposition of the specific polysaccharide of Type III Pneumococcus. For the sake of simplification, this culture will be referred to as the "S III bacillus."

2. *Description of the Culture.*—A morphological description of the organism is rendered difficult by its pleomorphism. In the mineral medium containing small amounts of specific substance (0.002 per cent), the organism appears as a minute Gram-negative bacillus, at times smaller than the Pfeiffer bacillus. In this medium also, spore formation takes place within the first 24 hours, and the cells are completely autolyzed by the 3rd day.

The cultural characters of the bacillus are much the same when grown in mineral medium containing larger amounts of specific substance (0.2 per cent); under these conditions the cells, however, are much larger and appear more resistant to autolysis.

The organism is Gram-negative in the mineral medium but harder to decolorize when grown in plain broth or on agar. At all stages, the cultures exhibit metachromatic granules, and in older cultures these granules appear as chains of minute coccus forms within the empty cell

membrane. The organism grows diffusely in peptone solution. In this medium sedimentation of the growth occurs after several days. On plain nutrient agar, free of dextrose, growth occurs in the form of small whitish colonies, 2 mm. in diameter, circular, slightly raised, umbilicated, with entire edge and fairly smooth surface. When grown in plain broth or in peptone solution, the organism appears as a fairly large bacillus, actively motile by means of peritrichous flagellae, the young cells measuring 2 to 3μ by 0.5μ . Short chains and especially diplo-forms are often observed. Organisms growing in this medium do not autolyze readily; in fact it is difficult to cause disintegration of the cells even by repeated freezing and thawing. Older cultures show the presence of elongated, thread-like involution forms and spores appear in 4 to 5 days. They are polar, oval, and very much larger than the rods. The heat resistance of the spores was measured in the following experiment.

Experiment 6. Heat Resistance of Spores of the S III Bacillus.—Tubes containing 5 cc. of casein peptone broth were inoculated with 0.2 cc. of a 5 day old culture of the bacillus. The inoculated tubes were heated at different temperatures for different lengths of time as indicated in Table I; the presence or absence of growth after 10 days incubation at 37.5°C . is indicated in the same table.

The results of Experiment 6 indicate that the spores of the organism resist heating for 30 minutes at 75°C ., but are killed by boiling for 5 minutes.

The fermentative ability of the specific organism was determined by growth in the basic mineral media to which various sugars, glucosides, and alcohols were added in concentration of 1 per cent. No gas was formed in any of these media and slight production of acid was observed only in the presence of dextrin, galactose, lactose, maltose, salicin, and trehalose.

Of particular interest is the action of glucose on the growth of this organism. It has been found that the addition of this sugar, to an otherwise favorable medium, exerts a decidedly inhibiting action on the development of growth.

In all media thus far tested, the organism is strictly aerobic, no growth occurring under anaerobic conditions.

In media containing, in addition to the capsular polysaccharide, other nutrients such as peptone, the decomposition of the specific

substance is much delayed. The delayed decomposition under these conditions is probably attributable to the so called sparing action of the peptone, which serves as a more readily available source of energy. As an illustration of this fact, it may be mentioned that, in peptone broth and in mineral medium containing a concentration of capsular polysaccharide equivalent to that naturally present in an autolysate of a bouillon culture of Type III *Pneumococcus*, the rate of decomposition of the specific substance is much slower in both the peptone broth and the autolysate than it is in the mineral solution, although growth develops more abundantly in the peptone-containing media.

TABLE I

Heat Resistance of the Spores of the S III Bacillus

Time of exposure	Growth in casein peptone broth after heating inoculum as indicated						
	70°C.	75°C.	80°C.	85°C.	90°C.	95°C.	100°C.
<i>min.</i>							
5	+	+	+	+	+	+	—
10	+	+	+	—	—	—	—
15	+	+	—	—	—	—	—
20	+	+	—	—	—	—	—
30	+	+	—	—	—	—	—

+ Refers to presence of growth after 10 days incubation.

— Refers to absence of growth after 10 days incubation.

It was of special interest to test the action of the organism on the specific polysaccharides of other types of *Pneumococcus* and on other polysaccharides of bacterial and plant origin.

Experiment 7. Specificity of the Action of the S III Bacillus.—The specific polysaccharides of *Pneumococcus* Types I, II, and III, Friedländer bacilli, Types A, B, and C (11), and *Hemophilus influenzae* Type a (12), and gum arabic were added to the basic mineral medium at pH 7.5, inoculated with 0.1 cc. of a young culture and incubated aerobically at 37.5°C. Decomposition of the different polysaccharides was tested by the serological method, using in each instance the specific antiserum, except in the case of gum arabic which was tested against *Pneumococcus* Type III antiserum (13) (see Table II).

The results of Experiment 7 emphasize the extraordinary specificity of the action of the organism on the specific polysaccharide of Type III

Pneumococcus. It is worth noting in particular that the organism does not decompose gum arabic even though this substance reacts in Type III antiserum.

The saprophytic nature of the S III bacillus is shown by the fact that 1 cc. of a young active culture may be injected intraperitoneally into a mouse without affecting the animal.

The Enzyme

The preceding experiments have established the existence of a microorganism which during growth in a synthetic medium breaks

TABLE II

Specificity of the Action of the S III Bacillus

Origin of the polysaccharide	Concentration of polysaccharide	Specific precipitin reaction of cultures after incubation at 37°C. for	
		1 day	30 days
	<i>per cent</i>		
Pneumococcus Type I.....	0.002	+++	+++
Pneumococcus Type II.....	0.002	+++	+++
Pneumococcus Type III.....	0.002	—	—
Friedländer's bacillus Type A.....	0.002	+++	+++
Friedländer's bacillus Type B.....	0.002	+++	+++
Friedländer's bacillus Type C.....	0.002	+++	+++
<i>Hemophilus influenzae</i> Type a.....	0.02	+++	+++
Gum arabic.....	0.01	+++	+++

+++ = marked precipitate formed in the corresponding specific antiserum.

— = negative precipitin reaction, indicating complete decomposition of the polysaccharide.

down the capsular polysaccharide of Type III Pneumococcus. It seemed possible that from cultures of this bacillus a soluble enzyme might be extracted which would decompose the specific substance in the absence of the bacterial cells.

Experiment 8. The Extraction of a Soluble Enzyme Capable of Decomposing the Specific Polysaccharide of Pneumococcus Type III.—The mineral medium (pH 7.5) containing 0.01 per cent of Pneumococcus Type III specific polysaccharide was seeded with a heavy inoculum of the S III bacillus and incubated at 37.5°C.

The specific substance was completely decomposed after 24 hours incubation;

at this time, when microscopic examination showed large numbers of well formed cells with only a few spores, 10 cc. of the culture were removed and passed through an N Berkefeld filter. The filtrate was designated Preparation 3-a. After 5 days further incubation, microscopic examination of the original culture showed that all the cells were lysed; the autolyzed culture was then passed through an N Berkefeld filter and the filtrate designated Preparation 3-b. Test tubes containing 1 cc. of a 0.001 per cent solution of specific substance (pH 7.5) received varying amounts of Preparations 3-a and 3-b; the mixtures were made up to a volume of 1.5 cc. and incubated at 37.5°C. for 18 hours in the presence of toluene to prevent any bacterial action. The precipitin test for the presence of specific substance was made after 18 hours incubation. The results of these tests are recorded in Table III.

TABLE III

The Decomposition of Type III Capsular Polysaccharide by Sterile Culture Filtrates of the S III Bacillus before and after Autolysis

Amount of filtrate cc.	Specific precipitin reaction of mixtures of substrate and culture filtrate	
	Before autolysis 3-a	After autolysis 3-b
0.5	+++	—
0.1	+++	+
0 (control)	+++	+++

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus sign indicates no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

The results of Experiment 8 show that 1 cc. of a 0.001 per cent solution of specific polysaccharide was completely decomposed by 0.5 cc. of the filtrate of an autolyzed culture (Preparation 3-b). On the contrary, 0.5 cc. of the filtrate of a young culture (Preparation 3-a) did not affect the specific substance. It is apparent, therefore, that the soluble principle responsible for the decomposition of the specific substance is released only after lysis of the cells. This experiment also seems to justify the assumption that the S III bacillus, when grown on a synthetic medium containing the specific polysaccharide, gives rise to a soluble endocellular enzyme capable of decomposing this substance.

Several enzymes are known to be extremely heat-resistant, most of them, however, are thermolabile and it was interesting to determine

the heat resistance of the active principle responsible for the decomposition of the specific substance in autolytic extracts of the S III bacillus.

Experiment 9. Inactivation of the Specific Enzyme by Heat.—Different lots of Preparation 3-*b* were heated at 50°, 55°, 60°, 65°, 70°, and 75°C. for 10 minutes and added in amounts of 0.1 cc. and 0.5 cc. to 1 cc. of 0.001 per cent solutions of SSS III. The mixtures were incubated for 18 hours at 37.5°C. in the presence of toluene and tested at that time for the presence of the specific polysaccharide by the precipitin reaction with Type III serum (see Table IV).

TABLE IV
Heat Inactivation of the Specific Enzyme

Enzyme heated 10 min. at	Specific precipitin reaction of mixture of enzyme and substrate
°C.	
50	—
55	—
60	+
65	+++
70	+++
75	+++

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

These results indicate that the soluble principle responsible for the decomposition of the capsular polysaccharide is inactivated after exposure for 10 minutes at 60-65°C.

Experiment 10. Activity of the Enzyme under Aerobic and Anaerobic Conditions.—Two tubes containing 2 cc. of a 0.02 per cent solution of SSS III received 2 cc. of active enzyme Preparation 3-*b*. One of the tubes was incubated at 37.5°C. under aerobic conditions, and the other under anaerobic conditions (Brown jar). After 24 hours incubation, the mixtures containing active enzyme and polysaccharide were tested for the presence of specific substance and it was found that the test had become negative in both tubes.

It has been shown previously that the organism is strictly aerobic and does not decompose the specific polysaccharide under anaerobic conditions. This experiment shows, however, that the soluble prin-

ciple, when extracted from the cells, decomposes the specific substance equally well under both aerobic and anaerobic conditions.

These results also indicate that the soluble principle is not of the nature of an oxidative enzyme, since there was no hydrogen acceptor in the mixture incubated under anaerobic conditions. Preliminary results, which indicate the presence of reducing sugars following the decomposition of the specific substance by the soluble principle, suggest that the action is one of hydrolysis.

It has been shown previously (Experiment 7) that of the polysaccharides thus far tested the specific substance of Type III *Pneumococcus*

TABLE V

Specificity of the Action of the Enzyme Derived from the S III Bacillus

Origin of the polysaccharide	Concentration of the polysaccharide	Specific precipitin reaction of enzyme substrate mixture incubated at 37°C.	
		Aerobically	Anaerobically
	<i>per cent</i>		
<i>Pneumococcus</i> Type I.....	0.002	+++	+++
<i>Pneumococcus</i> Type II.....	0.002	+++	+++
<i>Pneumococcus</i> Type III.....	0.002	—	—
Friedländer's bacillus Type A.....	0.002	+++	+++
Friedländer's bacillus Type B.....	0.002	+++	+++
Friedländer's bacillus Type C.....	0.002	+++	+++
<i>Hemophilus influenzae</i> Type <i>a</i>	0.02	+++	+++
Gum arabic.....	0.01	+++	+++

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

is the only one to be decomposed by the S III bacillus. The following experiment demonstrates that the same extraordinary specificity applies to the activity of the enzyme in cell-free filtrates of autolyzed cultures.

Experiment 11. Specificity of the Action of Enzyme Extracted from the S III Bacillus.—To different portions of enzyme Preparation 3-b were added the specific polysaccharides of *Pneumococcus* Types I, II, and III, Friedländer bacilli Types A, B, and C (11), *Hemophilus influenzae* Type *a* (12), and also gum arabic (13). The mixtures were incubated aerobically and anaerobically and tested for the presence of the polysaccharide after 1 month incubation at 37.5°C. (see Table V).

The results of Experiment 11 indicate that the soluble principle extracted from the bacterial cells is as specific in its action as is the living organism itself, decomposing only the capsular polysaccharide of Type III Pneumococcus.

Since it was proposed to test the effect of this specific enzyme on the course of pneumococcus infection in experimental animals, it was of interest to establish the influence of normal serum on the rate of decomposition of the capsular polysaccharide by the soluble principle.

Experiment 12. The Influence of Normal Serum on the Rate of Decomposition of the Capsular Polysaccharide by the Specific Enzyme.—To each of three tubes

TABLE VI

Influence of Normal Serum on the Rate of Decomposition of the Capsular Polysaccharide by the Bacterial Enzyme

Incubation period <i>hrs.</i>	Bacterial extract + specific substance +		
	Salt solution	Rabbit serum	Beef serum
0	++++	++++	++++
1	+++	+++	+++
2	+	++	+
3	±	+	±
4	—	±	—
5	—	—	—

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

containing 3 cc. of enzyme Preparation 3-*b* and 3 cc. of 0.002 per cent solution of SSS III, were added respectively 2 cc. of normal rabbit serum, 2 cc. of normal beef serum, and 2 cc. of physiological salt solution, the last serving as control. The tubes were incubated at 37.5°C. and samples of the mixtures were removed from time to time to follow the progress of the decomposition of SSS III, as measured by the specific precipitin reaction (see Table VI).

The results of this experiment indicate that the rate of decomposition of the Type III polysaccharide by the specific enzyme is not affected by the presence of normal beef or rabbit serum in the mixture.

It was of interest to devise a method for measuring the concentra-

tion or at least the comparative activities of different enzyme preparations. One possible method was to compare the rate of decomposition of a given concentration of specific substance by equal amounts of different preparations. A second method was to determine whether the minimum amount of bacterial enzyme capable of decomposing a given amount of specific substance—independently of time—would vary from one preparation to another, in other words, whether there existed a definite quantitative relationship between total amount of substrate decomposed and amount of active enzyme used. The following experiment was planned to compare the rate of decomposition

TABLE VII
The Rate of Decomposition of Specific Substance by Two Different Enzyme Preparations

Incubation period <i>hrs.</i>	Specific precipitin reaction of enzyme-substrate mixture	
	Prep. 3-b	Prep. 4-a
1	++++	++
2	+++	++
3	++	—
4	+	—
5	—	—

Plus signs indicate amount of precipitate formed in Type III antiserum.
Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

tion of the same amount of specific substance by two different enzyme preparations.

Experiment 13. Comparison of the Activity of Different Enzyme Preparations as Measured by the Rate of Decomposition of Capsular Polysaccharide.—Two preparations (3-b and 4-a) were compared. The former was the filtrate obtained from an autolyzed culture grown in a mineral medium containing 0.01 per cent of SSS III; 4-a was the filtrate of a culture grown in 0.2 per cent SSS III. These two preparations were added in amounts of 5 cc. to 5 cc. of a 0.002 per cent SSS III in buffer pH 7.5. The mixtures were incubated at 37°C. with toluene, and precipitin tests were made from time to time to follow the disappearance of the SSS III (see Table VII).

Table VII shows that when the time required for the decomposition of 1 cc. of a 0.002 per cent solution of specific polysaccharide by 1 cc.

TABLE VIII

Quantitative Relationship between Total Amount of Specific Polysaccharide Decomposed and Amount of Enzyme Used

Enzyme preparation		Specific precipitin reaction of enzyme-substrate mixture incubated for			
No.	Amount	1 hr.	6 hrs.	12 hrs.	24 hrs.
	cc.				
1	2.0	+++	+	—	—
	1.0	+++	++	—	—
	0.5	++++	++++	++	++
	0.2	++++	++++	+++	+++
	0.1	++++	++++	++++	++++
	0.05	++++	++++	++++	++++
	0.02	++++	++++	++++	++++
	0.01	++++	++++	++++	++++
3-b	2.0	++	—	—	—
	1.0	+++	+	—	—
	0.5	++++	++	—	—
	0.2	++++	++	+	+
	0.1	++++	++++	++++	++
	0.05	++++	++++	++++	++++
	0.02	++++	++++	++++	++++
	0.01	++++	++++	++++	++++
4-a	2.0	+	—	—	—
	1.0	+++	—	—	—
	0.5	+++	—	—	—
	0.2	++++	++	—	—
	0.1	++++	++	—	—
	0.05	++++	++++	+	+
	0.02	++++	++++	++	++
	0.01	++++	++++	+++	++++

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum, showing complete decomposition of the specific polysaccharide.

of the bacterial extract is used as a measure of the activity, enzyme Preparation 4-a is much more active than Preparation 3-b. This

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method of titration makes it possible to compare the relative activity of different enzyme preparations.

The following experiment was planned to compare the total amount of specific substance decomposed by given amounts of different enzyme preparations after different incubation periods.

Experiment 14. Titration of Activity of Different Enzyme Preparations as Measured by the Total Amount of Specific Substrate Decomposed.—Three preparations were compared. Preparation 1 was the filtrated autolysate of a culture grown in mineral medium containing 0.001 per cent SSS III; Preparations 3-b and 4-a were the same as described in the preceding experiment.

The enzyme preparations were added in amounts of 1 cc., 0.5, 0.2, 0.1, 0.05, 0.02, 0.01 cc. to 2 cc. of a 0.001 per cent solution of SSS III; the mixtures were made up to a volume of 3 cc. and were incubated with toluene at 37.5°C. 0.5 cc. samples were taken out from time to time to test for the disappearance of SSS III as determined by the precipitin reaction in Type III antipneumococcus serum.

The results recorded in Table VIII indicate that with a given amount of enzyme the maximum amount of decomposition of specific polysaccharide is reached after 12 hours incubation at 37.5°C. and that no further decomposition takes place thereafter. It appears in this particular instance that it required more than 1 cc. of Preparation 1 to decompose 2 cc. of the standard solution of Type III polysaccharide, whereas the same result was obtained with little more than 0.2 cc. of Preparation 3-b and 0.05 cc. of Preparation 4-a.

These results indicate that after a definite incubation period, the total amount of specific substrate decomposed bears a quantitative relationship to the concentration and activity of the enzyme preparation used. As a result of these experiments, it has been found that a convenient method of titrating the activity of an enzyme preparation is to determine the minimum amount that would decompose 1 cc. of a 0.001 per cent solution of specific capsular polysaccharide in 18 hours at 37.5°C.

DISCUSSION

The technique of isolation, and the biological characteristics of the bacillus described in this paper illustrate the great possibilities of the "starvation" method for stimulating certain potential properties of microorganisms. Although limited in its ability to utilize carbohy-

drate other than the capsular polysaccharide of Type III Pneumococcus, this organism grows rapidly and abundantly on ordinary media, plain broth, peptone solution, and casein hydrolysate. When grown on these media, however, the presence of other more readily available nutrients exerts a sparing action on the capsular polysaccharide.

When first isolated, the organism required 10 days to decompose the specific substance in concentration of 0.002 per cent; in its present state of activity, it decomposes a 0.01 per cent concentration of the polysaccharide in 24 hours. By repeated transfers in the specific medium this potential property has been greatly enhanced. It seems likely that this increase in activity is associated with an increased elaboration of the specific enzyme. Further data concerning this point will be presented later.

The fact that the growth of this organism is at least partly inhibited by the presence of glucose in the medium is unexplained but not a completely new phenomenon. There are on record at least two cellulose-decomposing species of bacteria the growth of which is inhibited by reducing sugars and especially glucose. It may be mentioned in passing that these organisms (*Sp. cytophaga* Hutchinson and Clayton (14), and "Y" bacillus Dubos (4)) are also extremely specific in their activities since cellulose is the only material on which it has been possible to grow them.

A comparison of the heat resistance of the spores and of the active enzyme extracted from the bacterial cells brings out the interesting fact that whereas the former resist heating at 95°C. for 5 minutes the latter is inactivated by 10 minutes exposure to 60°C.

It is also worth noting that although the organism is so strictly aerobic, the isolated enzyme responsible for the decomposition of the specific substance is equally active under anaerobic and aerobic conditions. It is likely that this soluble principle belongs to the group of hydrolytic enzymes.

Further work is in progress concerning the cultural conditions affecting the elaboration and activity of the enzyme. A fact of practical importance for experimentation is the possibility of titrating the activity of the enzyme *in vitro* by taking advantage of the quantitative relationship which exists between the total quantity of substrate decomposed and the amount of enzyme used.

Finally it may be mentioned that the first question which fostered this inquiry has been answered. The decomposition of the capsular polysaccharide of one of the specific types of *Pneumococcus* by a mild enzymatic action results in the loss of specific precipitability of this substance in antipneumococcus serum of the homologous type; that the polysaccharide, and not some impurity carried along with it, is responsible for type specificity, is once more proved, and probably beyond doubt.

The specificity of the types of *Pneumococcus* is illustrated also by the remarkably specific action of this enzyme which attacks only the capsular polysaccharide of Type III *Pneumococcus*; in fact this enzyme appears as specific as an antibody.

The answer to the two other questions mentioned at the beginning of this paper, namely the influence of the enzyme on the growth of Type III *Pneumococcus in vitro* and on the course of pneumococcus infection in experimental animals will be considered in subsequent papers.

SUMMARY

1. An organism has been isolated from peat soil which decomposes the specific capsular polysaccharide of Type III *Pneumococcus*.
2. The isolation has been made possible by the use of a synthetic mineral medium containing the specific polysaccharide as sole source of carbon. By repeated transfers in this medium the potential capacity of the organism to decompose the specific substance has been progressively increased.
3. The organism is a pleomorphic bacillus, motile and spore-bearing, exhibiting metachromatic granules; its reaction to the Gram stain varies according to the medium on which it is grown. It is strictly aerobic and grows well in plain broth and peptone solutions; it does not produce gas in any media and it forms small amounts of acid only on dextrin, galactose, lactose, salicin, and trehalose; its growth is inhibited by glucose.
4. The organism decomposes the capsular polysaccharide of Type III *Pneumococcus* aerobically, between pH 6.2 and 7.8, at room temperature and at 37.5°C., but not at 54°C. The decomposition of the specific substance is inhibited by the presence in the medium of other

nutrients, such as peptones, which act as a more readily available source of energy. The action of the organism is specific; it does not attack the soluble specific substance of Type I or Type II Pneumococcus, nor any of the other bacterial polysaccharides thus far tested.

5. The organism possesses an endocellular enzyme. This enzyme has been extracted by autolysis of the bacterial cells; in sterile solution it exhibits the same specific action as do the organisms from which it is derived, decomposing only the capsular polysaccharide of Type III Pneumococcus.

6. This enzyme decomposes the Type III specific polysaccharide under anaerobic as well as under aerobic conditions; it is inactivated at 60–65°C.; the rate of decomposition of the specific substance is not affected by the presence of normal serum.

7. There exists a quantitative relationship between the total amount of specific substance decomposed and the amount of enzyme preparation used; the existence of this relation makes it possible to express the activity of a given enzyme preparation in terms of the minimal amount required for the complete decomposition of a given amount of specific substance.

8. The specific decomposition of the capsular polysaccharide of Type III Pneumococcus, by the organism as well as by the enzyme it produces, illustrates once more the specificity of the types of Pneumococcus and confirms the fact that the capsular polysaccharides, and not some impurities carried along with them, are responsible for type specificity.

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THE PROTECTIVE ACTION OF A SPECIFIC ENZYME AGAINST TYPE III PNEUMOCOCCUS INFECTION IN MICE

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PLATE 4

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The cultural characteristics of a bacillus capable of decomposing the capsular polysaccharide of Type III Pneumococcus were described in the preceding paper (1). The decomposition of the specific carbohydrate was shown to be due to the action of an intracellular enzyme which may be extracted in active form from the bacilli. It was pointed out that the enzyme acts only on the capsular polysaccharide of Type III pneumococci and does not affect the specific carbohydrates of certain other encapsulated bacteria. In this respect, the enzyme exhibits a selective action which is as unique in its specificity as is the serological reaction between the capsular polysaccharide and homologous antibody. Furthermore, it was shown that the breaking down of the complex sugar by the enzyme is accompanied by a loss of the serological specificity of the carbohydrate. This form of decomposition by a biological agent represents a splitting which is less drastic than that accomplished by chemical hydrolysis with acid and heat. The fact that the breaking down of the sugar molecule is, in each instance, accompanied by the loss of its immunological specificity furnishes convincing evidence that the capsular polysaccharide itself is the substance responsible for the type specificity of pneumococci. The observations recorded in the preceding paper concerned the action of the enzyme on the chemically purified polysaccharide removed and separate from the bacterial cells. In its native state the capsular substance forms a morphological structure which conditions the antigenic and serological reactions of the cell as a whole, as well as its power to invade and multiply in the animal body. It was of special interest, therefore, to ascertain what effect this specific enzyme

would have upon the encapsulated cells growing *in vitro* and *in vivo*; whether in a medium containing the active enzyme Type III pneumococci would fail to grow, or would grow merely deprived of their capsules; whether in the body of a susceptible animal the administration of the enzyme would in any way modify the course of experimental infection with virulent Type III pneumococci. It is with these two questions that the present paper deals.

Before discussing the experimental results to be reported in this paper, it may be well at this point to mention briefly the observations of other investigators who have attempted to modify or inhibit the activity of bacteria by the use of certain enzymes.

Reference has already been made in previous papers (1-3), to the work of Toenniessen (4) who isolated in the form of galactan a gum-like material from an encapsulated strain of Friedländer's bacillus. Of special interest in this regard is his observation on the utilization of this capsular substance by *Bacillus vulgatus*, when both organisms were grown together in symbiosis. Under these conditions he observed that the Friedländer bacilli progressively lost their capsules, although the viability of the decapsulated forms was in no way impaired. This investigator attached no immunological significance to the capsular carbohydrate, nor did he attempt to apply the principle involved in this symbiotic relationship to the problems of experimental infection. Rather, Toenniessen regarded the substance of the capsule as a reserve food stuff, similar to starch in plants and glycogen in animals. From this viewpoint, however, he emphasized the significance of the capsule as a physiologically important part of the bacterial cell and showed that it may be decomposed by an unrelated species of microorganism.

In 1910 Vaudremer (5) pointed out that tuberculin, when added to filtered extracts of *Aspergillus fumigatus*, loses in great part its activity. This decomposition he attributed to the presence of various enzymes in the unheated extracts. He also showed that tubercle bacilli were modified by maceration in extracts of the fungus, and that under these conditions they became granular and less acid-fast. The injection of the bacilli, altered by growth in the fungus extracts, served to stimulate in animals an increased resistance to subsequent infection with virulent tubercle bacilli. Filtered extracts of the *Aspergillus* alone were used in the treatment of guinea pigs previously infected with tubercle bacilli. In these instances, the progress of the disease was often much retarded and the life of the treated animals prolonged. When Vaudremer had shown that unheated extracts of *Aspergillus fumigatus* were able to destroy tuberculin, to diminish the acid resistance of tubercle bacilli, and to retard infection in experimental animals, he applied the same methods to the treatment of tuberculosis in man.

That tubercle bacilli lose their acid fastness after 3 months incubation in a

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culture of an unidentified mold was also observed by Machado (6) who traced the action to the activity of the fungus enzymes.

The suggestion that the lipoid substances of tubercle and lepra bacilli may be acted upon by the esterases of the tissues is found in the observations of Citron and Reichen, Peritz and others (7), who have expressed the view that an increased content of these enzymes in the serum of patients is associated with an increased resistance to infection. The relative resistance of the skin to tuberculosis, although as Sexsmith and Petersen (8) suggest this resistance cannot be due wholly to the activity of this ferment since lepra bacilli, also rich in lipoids, invade the skin. The observation by Robinovitch, Stiles, and Payne (9) that tuberculosis of the pancreas is relatively uncommon in tuberculous animals led these workers to attempt to influence the disease in experimentally infected guinea pigs by the injection of pancreatic extracts containing active lipase.

The active principle, pyocyanase, present in autolysed cultures of *B. pyocyaneus* was found by Emmerich and his associates (10) to bring about rapid dissolution of many different species of living bacteria. They considered the active agent to be an enzyme belonging to the class of nucleases. Active preparations of pyocyanase were used in the treatment of animals experimentally infected with anthrax bacilli. The protection afforded by pyocyanase was attributed to the direct action of the enzyme on the infecting bacilli.

EXPERIMENTAL

Culture.—Young, plain broth cultures of a strain of Type III *Pneumococcus* were used in the following experiments. The virulence of the strain, maintained by repeated mouse passage, was such that 0.00000001 cc. of culture injected intraperitoneally into white mice caused death within 36 to 72 hours. The dilutions of the culture were made in such manner that the infecting dose was always contained in a constant volume of 0.5 cc.

Enzyme.—Filtered solutions of the enzyme extracted from the S III bacillus were prepared as described in the preceding paper.

Protection Tests.—White mice, 18 to 20 gm. in weight, served as test animals. All injections were made intraperitoneally and the technique commonly used in protection tests with antipneumococcus serum was employed. Unless otherwise stated, the infecting organisms and enzyme were injected simultaneously. In all instances the virulence of the culture was controlled by injecting normal mice with minimal amounts of the culture alone as shown in the protocols.

I. Action of the Enzyme on Encapsulated Type III *Pneumococci* in Vitro

Reference has already been made to the observations of Toenniesen (4) on the disappearance of the capsules of Friedländer bacilli when

these organisms are grown in symbiosis with *B. vulgatus*. Similar relationships have been found to exist in the case of the symbiotic growth of an encapsulated strain of Type III Pneumococcus together with the enzyme-producing bacillus. The specific enzyme elaborated by the bacilli brings about the decomposition of the capsular polysaccharide formed by the Pneumococcus. In symbiotic cultures of these two organisms, the pneumococci lose their agglutinability in type-specific serum, and the soluble specific substance can no longer be demonstrated free in the culture fluid. Obviously these results are conditioned by certain variable factors, such as the relationship between the rate of decomposition and the rate of production of capsular substance, and the maintenance of cultural conditions suitable for the physiological activity of both species of microorganisms. However, there is no difficulty in demonstrating the reaction when, instead of the living bacilli, a sterile extract of the enzyme itself is added in suitable concentration to the culture medium. Under these conditions the decomposition of the capsular polysaccharide by the enzyme can be demonstrated during growth of the pneumococci, by failure of the culture to react specifically when added to Type III antipneumococcus serum.

Experiments of this nature reveal the fact that the enzyme by itself is neither bactericidal nor bacteriolytic; that by decomposing the specific carbohydrate, the enzyme merely deprives the bacteria of their capsules without impairing the viability of the cells. It is also evident that the action of the enzyme does not result in a loss of the function of elaborating the capsular substance, since organisms so treated regain their capsule and form the specific polysaccharide when transferred to a medium free of the enzyme.

The experiments reported in the preceding paper (1) showed that an enzyme derived from an unrelated species of microorganisms acts upon the Type III capsular polysaccharide of Pneumococcus when this substance, separated from the bacterial cells, is used as the specific substrate. The experiments just described afford evidence that the enzyme also brings about the decomposition of this same capsular material in the native form in which it exists as a structural part of the living organism. Since it has been shown that the enzyme has a definite effect upon the capsular component of the cell growing *in*

vitro, the question naturally arose, whether it would exert a similar action on the encapsulated forms growing in the animal body; to seek the answer to this question the following experiments were designed.

II. The Action of the Enzyme on Encapsulated Type III *Pneumococci* *in Vivo*

Specificity of the Action of Enzyme in Mice.—To determine whether the enzyme would protect mice against infection, and to what extent the specificity exhibited by the enzyme *in vitro* would be reflected in

TABLE I
Specificity of the Protective Action of Enzyme

Infecting dose of <i>Pneumococcus</i> cc.	Enzyme (Lot 4-c) 0.5 cc.			No enzyme		
	<i>Pneumococcus</i> Type I	<i>Pneumococcus</i> Type II	<i>Pneumococcus</i> Type III	Virulence controls		
				Type I	Type II	Type III
0.1	—	—	S	—	—	—
0.01	—	—	S	—	—	—
0.001	—	—	S	—	—	—
0.0001	D 20	D 34	S	—	—	—
0.00001	D 24	D 34	S	D 22	D 36	D 34
0.000001	D 34	D 34	S	D 34	D 36	D 34
0.0000001	—	—	—	D 34	D 20	D 72

S = survived.

D = death of animal; the numeral indicates the number of hours before death, or the time at which the animal was found dead.

— = not done.

its action in animals, three separate groups of mice were infected with *Pneumococcus* Types I, II, and III, each animal receiving at the same time a constant amount of the Type III specific enzyme. The experiment was carried out as follows:

The technique of the protection test was the same as that already described. Three mice were injected intraperitoneally with 10^{-4} , 10^{-5} , and 10^{-6} cc. of a broth culture of Type I *Pneumococcus*; three other mice received the same amounts of a culture of Type II; six mice were given much larger doses of a culture of Type III pneumococci ranging in amounts from 10^{-1} to 10^{-6} cc. Simultaneously with the bacteria each mouse of all three groups received 0.5 cc. of the same preparation of

enzyme (Lot 4-a). The virulence of the three types of pneumococci was controlled in each instance by the infection of normal mice with 10^{-5} , 10^{-6} , and 10^{-7} cc. of the respective culture alone.

The results of this experiment are recorded in Table I. The evidence clearly shows that the enzyme afforded mice protection against 1 million times the fatal dose of a virulent culture of Type III Pneumococcus. It is further apparent that the protective action is type-specific, since mice receiving the same enzyme but infected with amounts as small as 0.000001 cc. of a culture of a heterologous type promptly succumbed to infection. Just as in the test tube, the enzyme acts only on the Type III polysaccharide, so in the animal body it is effective only against infection with Type III Pneumococcus.

Heat Inactivation of the Enzyme.—Experiments previously reported (1) showed that the activity of the enzyme, as measured by the decomposition of the free carbohydrate, was destroyed by exposure to a temperature of 60°C . for 10 minutes. In the following experiment, an active preparation of the enzyme was heated, at a temperature known to destroy completely its action in the test tube, in order to determine the effect of heat upon the protective power of enzyme in mice.

5 cc. of enzyme preparation (Lot 4-a) were heated in a water bath at 70°C . for 10 minutes. Each of three mice was injected intraperitoneally with 0.5 cc. of the *heated* enzyme together with a culture of Type III Pneumococcus in amounts of 10^{-4} , 10^{-5} , and 10^{-6} cc. respectively. Each of five other mice received similar amounts of the same preparation of the *unheated* enzyme simultaneously with an amount of the cultures varying from 10^{-1} to 10^{-5} cc., respectively. Three mice infected with culture alone in doses of 10^{-5} , 10^{-6} , 10^{-7} cc. were used as virulence controls.

The results of this experiment show that the active principle responsible for the protection of mice against infection is destroyed by exposure to 70°C . for 10 minutes. Mice receiving the *heated* enzyme succumbed to infection with the smallest dose of culture used, 0.000001 cc. On the other hand, mice injected with an equal amount of the same enzyme *unheated* survived as much as 0.1 cc. of the same culture of which 0.0000001 cc. alone proved fatal for the untreated controls. The inactivation by heat of the protective power of the enzyme in the animal body, parallels the loss of its activity *in vitro* after exposure to

temperatures of 60°C. or higher. The fact that heat destroys the activity of the enzyme both *in vitro* and *in vivo*, supports the assumption that the same principle is involved in the mechanism of both reactions.

Relation between the Concentration and Protective Action of Enzyme.—Repeated tests have demonstrated that within the limits of the reaction capacity of the mouse, the protective action of the enzyme is a function of the concentration of the active principle in any given preparation. It is also apparent that the protection afforded by different

TABLE II
Inactivation of Enzyme by Heat

Pneumococcus Type III cc.	Enzyme (Lot 4-a) 0.5 cc.		No enzyme
	Unheated	Heated at 70°C. 10 min.	Virulence control
0.1	Survived	—	
0.01	Survived	—	
0.001	Survived	—	
0.0001	Survived	D 34	
0.00001	Survived	D 72	D 34
0.000001	—	D 72	D 34
0.0000001*	—	—	D 72

D = death of animal; the numeral indicates the number of hours before death.

— = not done.

* The inoculum at this dilution of culture (10^{-7} cc.) yielded 13 colonies on growth in blood agar.

preparations of the enzyme bears a relation to their capacity to decompose a known quantity of the capsular polysaccharide *in vitro*.

The comparative protective action in mice of two separate preparations, one of which was much less active *in vitro* is shown in Table III.

From Table III it is evident that Preparation 3, which was much less active than Preparation 4-a in decomposing the polysaccharide in the test tube, is also the less effective in the animal body. Although the results obtained by the two methods of titration are not wholly comparable, they indicate the existence of a correlation between the *in vitro* and *in vivo* activity of the enzyme. The comparison of these two

different preparations also suggests that the concentration of the enzyme in Preparation 3 is close to the minimum threshold value below which the enzyme ceases to function in protecting against even minimal infecting doses. From this and other similar experiments, the impression is gained that to be effective in animal protection, the concentration of enzyme in the body must be in excess so that the rate of decomposition is greater than the rate of production of the capsular substance by the living bacteria. The evidence also indicates that the greater the activity of the enzyme *in vitro* the greater is its protective

TABLE III

Protective Action of Enzyme against Infection with Pneumococcus Type III

Difference in protection titre of two preparations of enzyme which show marked differences in their activity *in vitro*.

Pneumococcus Type III	Enzyme 0.5 cc.		No enzyme
	Lot 3	Lot 4-a	Virulence controls
cc.			
0.1	—	S	
0.01	D 45	S	
0.001	D 72	S	
0.0001	D 72	S	
0.00001	S 6 days	S	D 34
0.000001	S	S	D 34
0.0000001	—	—	D 72

S = survival of animal.

D = death of animal; the numeral indicates the number of hours before death.

— = not done.

action in mice. Both of these deductions, however, suffer the limitations imposed by the variations which occur in the cellular response of the host, as will be pointed out later in discussing the importance of phagocytosis in the protective reaction.

Titration of Protective Action.—The concentration of enzyme in any given preparation as measured by the protection test in mice may be ascertained in two ways; (1) by determining the protective action of varying amounts of enzyme against a fixed quantity of culture, and (2) by determining the maximum amount of culture against which a constant quantity of the enzyme will protect. The results of experi-

ments of this nature are given in Tables IV and V. Although in the present instance both methods of titration were not carried out on the same preparation, the examples given illustrate the general principle. Table IV shows that 0.1 cc. of enzyme preparation protected mice against 0.01 cc. of a virulent culture, an amount 1 million times greater than the minimal dose fatal for the normal controls.

TABLE IV
Titration of Protective Action of Enzyme against Infection with Pneumococcus Type III
1. Varying amounts of enzyme and constant amount of culture.

Enzyme (Lot 7)	Pneumococcus Type III (1 million fatal doses)		Virulence controls
	Amount	Result	
cc.	cc.		
1.0	0.01	S	
1.0	0.01	S	
0.5	0.01	S	
0.5	0.01	S	
0.25	0.01	S	
0.25	0.01	S	
0.1	0.01	S	
0.1	0.01	S	
0	0.000001 cc.		D 45
0	0.0000001 cc.*		D 35
0	0.00000001 cc.†		D 46

S = survival of animal. Observation period 10 days.
D = death of animal; the numeral indicates the number of hours before death of animal.
* Inoculum of this amount of culture (10^{-7} cc.) yielded 30 colonies in blood agar.
† Inoculum of this amount of culture (10^{-8} cc.) yielded 1 colony in blood agar.

The results presented in Table V illustrate the maximum amount of culture against which 1 cc. of enzyme preparation (4-a) protected mice. Under the experimental conditions, this particular preparation protected against infection with 0.2 cc. of culture, an amount which represents 2 million fatal doses. Death was delayed in mice infected with 0.3 and 0.4 cc. of culture, the animals surviving as long as 4 days. Large infecting doses of this order however apparently represent the

upper limit against which a single dose of the enzyme fails to protect. Whether repeated injections of enzyme during the course of the infection, or whether the perfecting of methods for concentrating and purifying the enzyme will enhance its protective action, cannot now be stated. Moreover the maintenance of an effective concentration of enzyme in the body during the course of infection appears necessary to achieve the maximum of protection.

In order to ascertain how long the enzyme remains active after injection into normal mice, the following experiment was carried out.

TABLE V

Titration of Protective Action of Enzyme, against Infection with Pneumococcus Type III

2. Constant amount of enzyme and varying amounts of culture.

Pneumococcus Type III	Enzyme (Lot 4-a)	
	Amount	Result
cc.	cc.	
0.5	1.0	D 18
0.4	1.0	D 4 days
0.3	1.0	D 4 days
0.2	1.0	S
0.1	1.0	S
0.01	1.0	S
0.000001	0	D 25
0.0000001	0	D 42

D = death of animal; numeral indicates number of hours before death.

S = survival of animal.

Mice were injected intraperitoneally with 1 cc. of a sterile solution of enzyme. At intervals thereafter varying from 20 to 43 hours, the mice were infected by the intraperitoneal injection of varying amounts of a virulent culture of Type III *Pneumococcus*.

The results given in Tables VI and VII show that there is a gradual diminution in the protective action of the enzyme after its injection into the animal body. To determine more accurately the length of time during which the enzyme remains active in normal animals will require further detailed study. From the evidence available at present, it seems not unlikely that the occasional death of an infected

TABLE VI

Protective Action of Enzyme against Infection with Pneumococcus Type III
Action of enzyme given 24 hours before and simultaneously with the infecting dose of culture.

Pneumococcus Type III cc.	Enzyme (Lot 7) 1 cc.		No enzyme
	Simultaneously	24 hrs. before	Virulence controls
0.2	S	D 6 days	
0.1	S	S	
0.01	S	S	
0.001	S	S	
0.0001	S	S	
0.000001	—	—	D 45
0.0000001	—	—	D 35
0.00000001	—	—	D 45

D = death of animal; the numeral indicates the number of hours before death.
S = survival of animal—observation period 10 days.
— = not done.

TABLE VII

Protective Action of Enzyme Given before Infection with Pneumococcus Type III
Action of enzyme given 20 to 43 hours before infection.

Pneumococcus Type III cc.	Enzyme (Lot 6) 1 cc.	Enzyme (Lot 4-a) 1 cc. given		Virulence controls
	20 hrs. before infection	24 hrs. before infection	43 hrs. before infection	Culture alone
0.1	S	D 24	D 48	
0.01	S	S	S	—
0.001	S	S	S	—
0.0001	S	S	S	—
0.00001	S	S	S	—
0.000001	S	S	S	—
0.0000001	—	—	—	D 46
0.00000001	—	—	—	D 46
				D 46

S = survival of animal.
D = death of animal; the numeral indicates the number of hours before death.
— = not done.

animal several days after the administration of a single dose of the enzyme (Table VII) may be attributable to the loss or inactivation of the active agent during the course of the infection, thereby allowing a few organisms to escape and reestablish themselves in the absence of an effective concentration of the protective principle.

Curative Action of the Enzyme in Mice.—The preceding experiments were designed to demonstrate the protective action of the enzyme when administered simultaneously with the infecting microorganisms. In a few experiments the enzyme was given several hours before the bacteria were injected, in order to ascertain the duration of the activity of enzyme in the animal body. The degree of protection afforded under these circumstances is noteworthy, when the susceptibility of mice to pneumococcus infection, and the virulence of the strain employed are taken into consideration. It seemed of even greater interest, however, to determine whether the enzyme would have any effect when injected into mice that had been previously infected with a fatal dose of culture. The following experiment illustrates the so called curative action of the enzyme in the presence of an infection already established at the time of treatment.

Nine normal mice were infected with 10^{-6} cc. of broth culture of a virulent strain of *Pneumococcus* Type III. 12 hours later three of these animals were given an intraperitoneal injection of 1 cc. of enzyme (Preparation 4-a). Three other mice of the infected group were treated with the same amount of enzyme 18 hours after the infecting organisms had been given. The remaining three mice received no enzyme and served as untreated controls. As further controls of virulence two normal mice were injected with the culture alone in amounts of 10^{-7} and 10^{-8} cc. respectively. To estimate the number of organisms, these inocula were plated in blood agar and the number of colonies developing were counted. 10^{-7} cc. of culture yielded twenty-five colonies in the poured plates, and 10^{-8} cc., three colonies.

The results of this experiment on the curative action of the enzyme (Table VIII) show that mice receiving a single injection of the enzyme 18 hours after the onset of infection, recovered, whereas the untreated controls all died. Under the experimental conditions, the survival of the treated animals represents recovery from an infecting dose of culture 100 times greater than that fatal for untreated mice. While the conditions of this test were not the most severe, the outcome indi-

cates that the enzyme is effective when administered early in the course of an infection which otherwise invariably proves fatal. From other experiments, evidence has been gained that the administration of the enzyme several hours after infection with larger amounts of culture may favorably influence the course of the infection in mice.

Mechanism of the Protective Action.—It was thought possible that some idea of the mechanism involved in the protective action of the enzyme in infected mice might be gained by following the course of the bacteremia by means of blood cultures, and by a study of the

TABLE VIII

Curative Action of Enzyme in Mice Infected with Pneumococcus Type III

Pneumococcus Type III (100 fatal doses)	Enzyme 1 cc. (Lot 4-c) given		Controls
	12 hrs. after infection	18 hrs. after infection	No enzyme
cc.			
0.000001	S	S	D 35
0.000001	S	S	D 59
0.000001	S	S	D 59
0.0000001*	—	—	D 84
0.000000001†	—	—	D 60

D = death of animal; the numeral indicates the number of hours before death.

S = survival of animal.

* = Inoculum of this dilution of culture (10^{-7} cc.) gave 25 colonies in blood agar.

† Inoculum of this dilution of culture (10^{-8} cc.) gave 3 colonies in blood agar.

cellular reactions in the peritoneal exudates of treated and untreated animals. A typical experiment follows:

Twelve mice were injected intraperitoneally with 10^{-2} cc. of a culture of *Pneumococcus* Type III, the virulence of which was such that 10^{-5} cc. caused death in from 36 to 72 hours. Six of the mice also received at the time of infection 0.5 cc. of an active preparation of the enzyme. The other six infected animals served as untreated controls. At hourly intervals following infection, one mouse of each group was sacrificed and autopsied. Cultures of the heart's blood were made in broth and on blood agar plates. Films of the peritoneal exudates were stained by the Gram method, and examined to compare differences in the morphology of the organism and in the occurrence of phagocytosis in the treated and untreated animals.

The results of blood cultures taken during the course of the infection show that the enzyme has a distinct effect in checking the bacteremia which invariably occurs in infected mice. In both the treated and untreated animals pneumococci are present in the blood stream within the 1st hour following infection. However, the subsequent course of events, with reference to the persistence of bacteremia, differs in the two series of animals. For, while the bacteria progressively increase in the blood of the control mice until death, they invariably diminish in numbers in the circulation of the treated animals until by the 4th or 5th hour they can no longer be demonstrated by culture. The sterilization of the blood under these conditions is objective evidence of the protective action of the enzyme.

The fate of the pneumococci under the influence of the enzyme is strikingly revealed by microscopic study of the peritoneal exudate during the course of the infection. Without giving in detail the results of the serial examinations at hourly intervals, the progress of events is shown in the accompanying photomicrographs which illustrate the differences in the cellular reactions of treated and untreated mice 2 and 4 hours after the injection of 1 million fatal doses of *Pneumococcus* Type III. Two hours after infection the peritoneal exudate of the untreated control mouse (Fig. 1) shows numerous well encapsulated cocci free in the fluid. In contrast to this, the pneumococci in the exudate of the enzyme-treated animal at this time (Fig. 2) are devoid of capsules and only the naked bacteria are visible, many of which are already engulfed by the polynuclear leucocytes. At the end of 4 hours, the number of encapsulated pneumococci have increased in the peritoneum of the untreated control (Fig. 3), there is no evidence of phagocytosis, and cultures of the heart blood indicate a progressively increasing bacteremia. In the treated mouse at the end of 4 hours (Fig. 4) only an occasional unencapsulated organism is seen outside the accumulated leucocytes and frequently at this time and almost invariably by the 5th hour, pneumococci are no longer demonstrable by blood cultures. These findings have been repeatedly confirmed in a series of similar experiments and they support the view that the protective action of the enzyme lies in its capacity to decompose the capsular polysaccharide of Type III *Pneumococcus*.

DISCUSSION

The present study emphasizes the importance of the capsule in the biological reactions of the pneumococcus. It is, indeed, a significant fact, that no matter whether one regards this organism from the viewpoint of type specificity, antigenicity, or its capacity to undergo variation, or whether, as in the present instance, one considers the pneumococcus with reference to its virulence and fate in the animal body, the one dominant factor influencing all these phenomena is the function of the cell to elaborate the specific capsular polysaccharide. These relationships, however, are not to be interpreted as meaning that virulence is dependent merely upon differences in the structural morphology of the bacterial cell. For it is a common observation that an encapsulated strain of *Pneumococcus* may be virulent for one species and not for another. However, it is equally true that the function of elaborating the specific capsular polysaccharide is most highly developed in pneumococci that are best adapted to growth in the animal body. From this point of view, virulence and capsule formation, although not causally related, are at least intimately associated. When the function of forming the capsular substance is suppressed or inhibited, as in the case of the R variants, or when, as in the present instance, although this function is unimpaired the capsule itself is destroyed by an enzyme, the naked bacteria are thereby exposed directly to attack by the phagocytes of the host.

In this sense, the action of the enzyme may be said to result in preparing the encapsulated bacteria for phagocytosis; not, as in the case of antibodies, by specific sensitization, but by the process of decapsulation. In the former instance, the reaction is an immunological one, whereby the capsular material is altered by union with the type-specific antibody; in the latter case, the reaction is a chemical one in which the capsular polysaccharide is actually decomposed by the enzyme. Although the mode of action of both these specific agents is different in each instance, the end result, so far as the fate of the microorganism is concerned, is the same in both cases.

It is of interest that although neither the enzyme nor the specific antibody is by itself bactericidal or bacteriolytic, yet each by reacting specifically with the capsular substance exposes the virulent organisms to the phagocytic action of the body tissues. The enzyme, like the

specific antibody, serves merely to initiate the protective reaction, the completion of which is ultimately dependent for its successful issue upon the effective cellular response of the host.

The present study also suggests that the capsule—long recognized as a defense mechanism on the part of virulent bacteria—is a decisive factor in determining the fate of pneumococci in the animal body, and that this structure is vulnerable to attack by specific agents other than antibodies.

SUMMARY

The bacterial enzyme which decomposes the purified capsular polysaccharide of Type III Pneumococcus *in vitro* also destroys the capsules of the living organisms growing in media and in the animal body.

Potent preparations of this same enzyme protect mice against infection with virulent Type III Pneumococcus. The protective action is type-specific.

The protective activity of the specific enzyme is destroyed by heat (70°C. for 10 minutes).

The enzyme remains in an effective concentration 24 to 48 hours after its injection into normal mice.

The enzyme has been found to exert a favorable influence on the outcome of an infection already established at the time of treatment.

A definite relationship has been found to exist between the activity of the enzyme *in vitro* and its protective power in the animal body.

The mechanism of the protective action is discussed with special reference to the relation between the decapsulation of the bacteria by the enzyme and the phagocytic response of the host.

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EXPLANATION OF PLATE 4

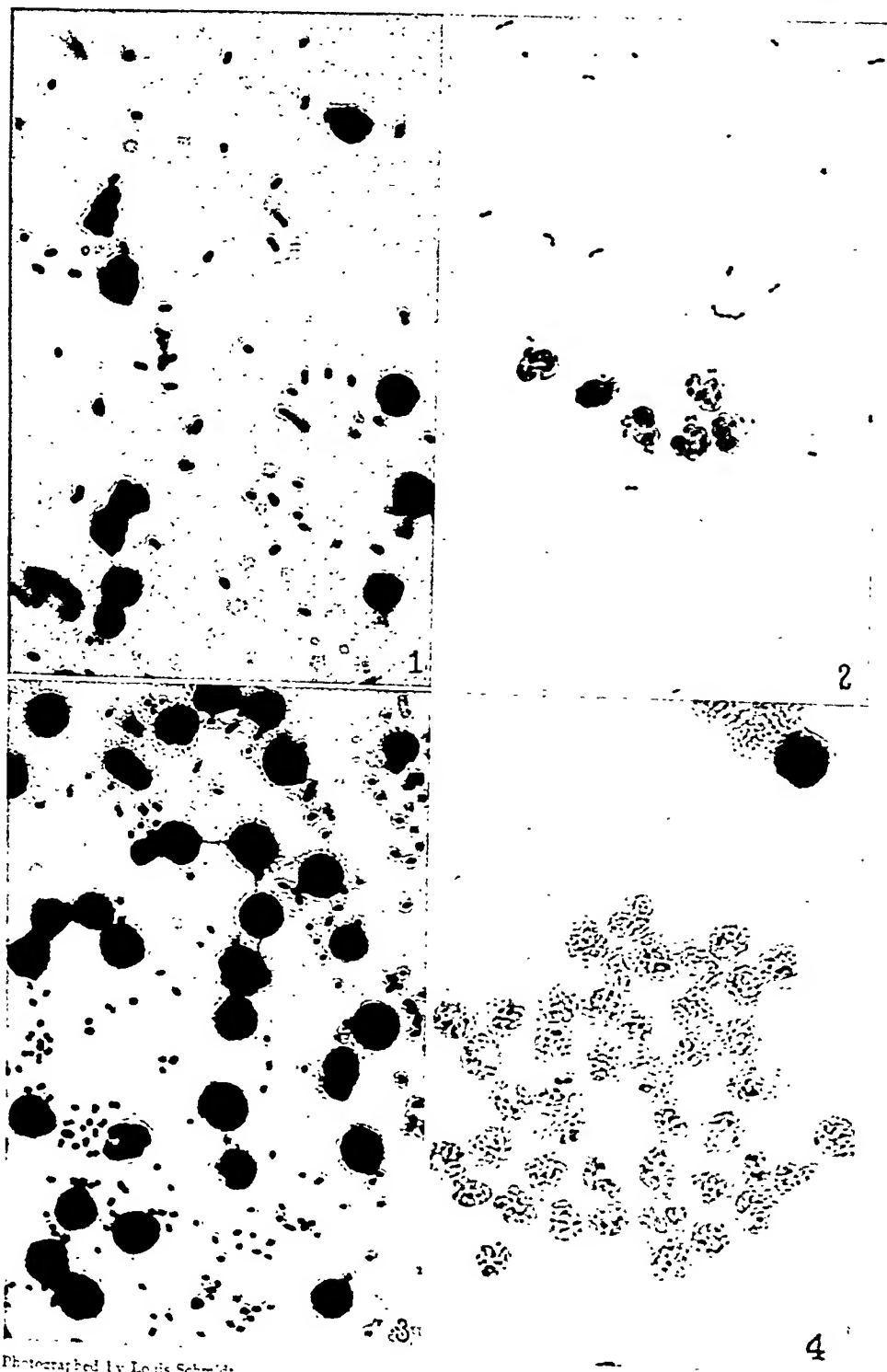
FIG. 1. Photomicrograph of a stained preparation of the peritoneal exudate of a mouse 2 hours after the intraperitoneal injection of 0.01 cc. of a virulent culture of Type III Pneumococcus. The bacteria show well defined capsules and no evidence of phagocytosis is seen. Many polymorphonuclear and a moderate number of mononuclear leucocytes are present. Gram stain, $\times 1000$.

FIG. 2. Photomicrograph of a corresponding preparation of the exudate of a mouse 2 hours after receiving the same amount of culture together with 0.5 cc. of a preparation of the specific enzyme. The bacteria are devoid of capsules. Polymorphonuclear leucocytes predominate and phagocytosis is evident. Gram stain, $\times 1000$.

FIG. 3. Photomicrograph of a stained film of the peritoneal exudate of a mouse 4 hours after injection with 0.01 cc. of culture alone. The bacteria are increased in number, encapsulated, and extracellular. The cellular elements are polymorphonuclear and mononuclear leucocytes in about equal numbers. Gram stain, $\times 1000$.

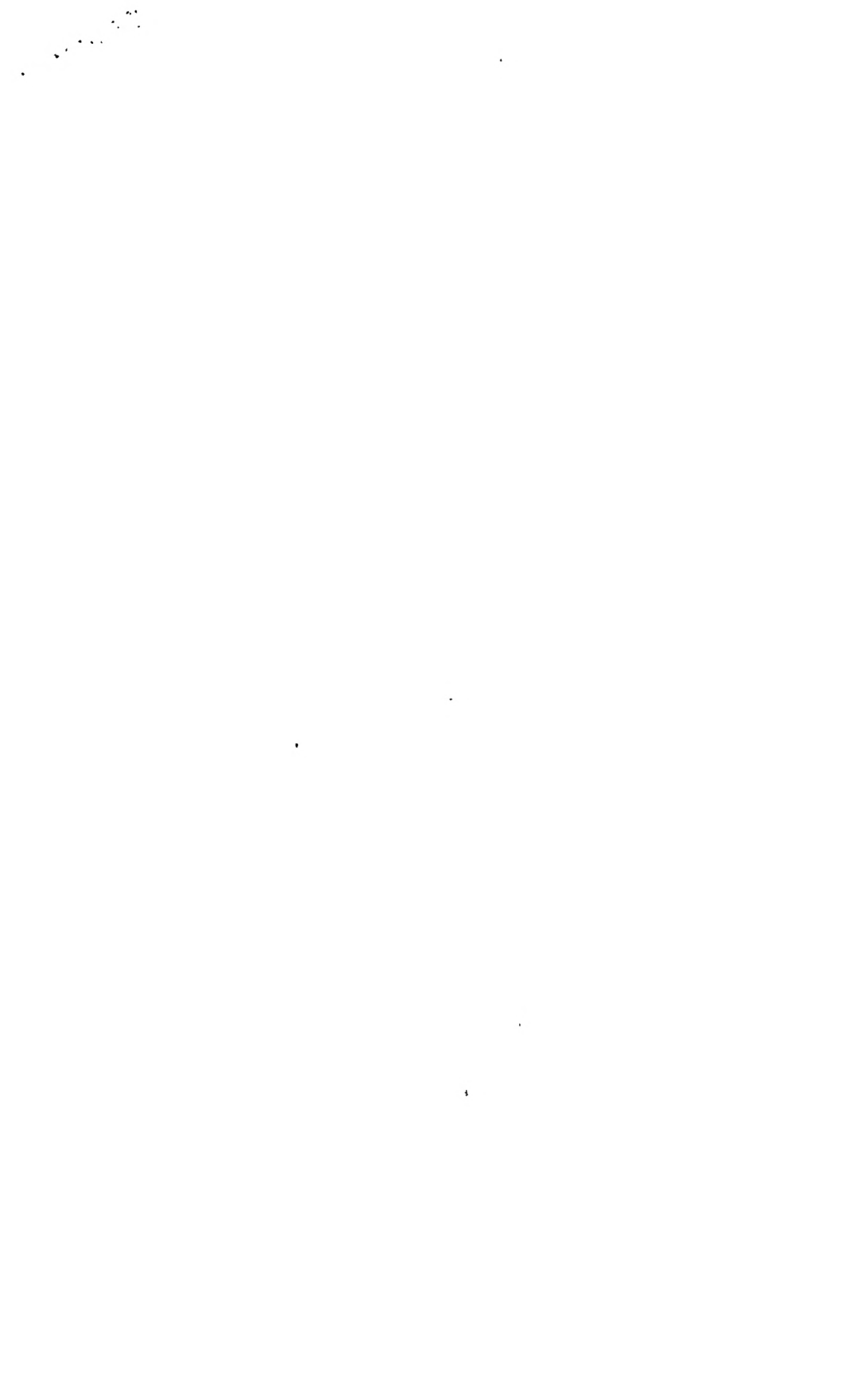
FIG. 4. Photomicrograph of a corresponding preparation of the exudate of a mouse 4 hours after receiving the same amount of culture together with 0.5 cc. of a preparation of the specific enzyme. Marked phagocytosis has occurred and only an occasional organism is seen outside the accumulated leucocytes, nearly all of which are of the polymorphonuclear type. Gram stain, $\times 1000$.

Differences in the density of the backgrounds of the four figures are due to the use of color screens in the photographic reproductions. This technique, however, alters none of the essential details observed in the original microscopic preparations.



Photographed by Louis Schmidt

(Avery and Dubos: Enzyme against Type III Pneumococcus)



PSITTACOSIS

I. EXPERIMENTALLY INDUCED INFECTIONS IN PARROTS

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PLATES 5 TO 7

(Received for publication, April 1, 1931)

The widespread outbreak of psittacosis in man in 1929-30 led to renewed interest in the disease which quickly resulted in the discovery of the facts that the causative agent is not Nocard's bacillus but a filterable virus (1) and that the incidence of laboratory infections is high. Because of these facts it became imperative that further work on the nature of the etiological agent and the mode of spread of the infection be undertaken.

Krumwiede and his coworkers (2) were among the first to show that the inciting agent of psittacosis is capable of passing filters and that mice are susceptible to the disease. Due to his ill health, however, and because of the development of psittacosis in several of his assistants, investigations so admirably begun by him had to be discontinued and two strains of the virus with which he was working were given to us for further study. The purpose of this paper and the three that follow is to present in detail investigations, already described in preliminary papers (3-5), concerning psittacosis experimentally produced in parrots, mice, rabbits, guinea pigs, and monkeys. Special attention has been paid to the mode of spread of the disease and to the pathological changes induced, depending upon the host and upon the portal of entry of the virus.

Methods and Materials

Virus—Krumwiede provided us with two strains of virus, one from a patient, the other from a parrot. Parrot Wenz C after feeding upon lung and spleen from a fatal case of psittacosis was transferred to our laboratory. Parrots N, O, and R were inoculated intramuscularly with spleen emulsion from Parrot M, and Parrot K was infected intramuscularly and intraorally with organ filtrate from Parrot

F. Following inoculation these birds were sent to The Rockefeller Institute. From examination of Text-fig. 1, it will be seen that the virus with which Parrots K, N, O, and R were inoculated came originally from a natural infection in Parrot 17. In Text-fig. 1, the doubly starred birds were handled only by Krumwiede and his coworkers, the singly starred parrots were inoculated by them and given to us, the birds without stars were seen and handled only by us.

Parrots.—Most birds used were Amazon parrots, while the remainder came either from Cuba or Mexico.

Inoculations.—Inoculations were effected by instillation of the virus in the nose and mouth, by pollution of the food and drinking water with the inciting agent, or by injection of the virus into the pectoral muscles.

Examination of Tissues.—The majority of the autopsies was performed immediately after the animals had died or after they had been sacrificed by means of chloroform. In all instances, aerobic and anaerobic cultures from various organs were made for the detection of ordinary bacteria. Sections from tissues fixed in Zenker's fluid and in 10 per cent formalin were stained with eosin and methylene blue or according to Wolbach's modification of Giemsa's method.

Measures Employed to Prevent Laboratory Infections.—At the time we began our work it had already become apparent that laboratory infections are a menace. Consequently measures were instituted to prevent as far as possible the spread of the disease to those actually working with the malady and to protect completely other individuals in the Institute.

Two rooms on the same floor that contained our laboratory and the central media rooms were placed at our disposal for the housing of animals. The openings into the animal rooms were so fixed that no cracks between the doors and windows and their frames, large enough to permit the passage of insects, remained. Furthermore, the doors and windows were doubly screened with coarsely and finely meshed wire. Thus the mechanical spread of the virus by mice and insects was absolutely prevented. One room housed the animals known to be infected, while the other was used for animals inoculated with material undergoing tests for the presence of virus. In the "clean room" the animals were caged in individual units and workers before going from one section to another washed their gloved hands with 5 per cent lysol.

All cages were cleaned and then sterilized with 5 per cent lysol 3 times a week as well as between experiments. Droppings and bedding were collected in large covered cans containing 5 per cent lysol. After the exterior of the cans had been washed with lysol and while the containers were still wet, they were conveyed to the incinerator into which the refuse was dumped and immediately burned. Animals to be autopsied were dipped, after death, in 5 per cent lysol and wrapped in towels moistened with the same solution before being taken to the laboratory. Finally, the floors were cleansed each day with lysol and allowed to remain wet.

The cleaning of the cages and rooms and the examination of the sick animals constituted the most dangerous duties connected with the work. This is true, because the virus probably enters man through the upper respiratory tract, *i.e.*,

the conjunctival sac, nose, and mouth. When the work was begun, however, this fact was not definitely known. Consequently a costume to prevent all modes of infection was devised. A photograph of this costume is shown in Fig. 1. The uniform consists of air-tight goggles, the lenses of which were treated with a patented preparation of soap and glycerol to prevent steaming, a mask made of several thicknesses of gauze to cover the nose and mouth over which was placed a respirator frame, a hood covering the head and shoulders (the goggles were held in the hood by firmly tied draw-strings), a surgeon's gown over which was placed a heavy rubber apron, heavy rubber obstetrical gloves reaching to the elbows, and heavy rubber boots long enough to cover the legs as far up as the knees. When handling the animals, the workers wore heavy leather gloves wet with lysol in order to prevent injury to the hands and destruction of the rubber gloves. As is well known it is almost impossible to prevent the inhalation of dried infectious material unless a gas mask is used. The costume employed by us was fairly efficacious chiefly because of the dead space created by the respirator frame placed between the gauze mask and hood. When working with the virus in the laboratory, we wore goggles, a gauze mask, a surgeon's gown, and rubber gloves. The parts of the costume made of cloth, upon being removed, were dipped in lysol solution before being sent to the laundry. Rubber portions of the costume were sterilized either by boiling or by immersion in lysol solution.

All containers with liquid media were inclosed in tin cans while being incubated. Petri dishes with cultures were sealed by means of strips of rubber cut from Ford inner tubes. These measures were used to prevent the entry of insects into the plates and the consequent spread of infection, and to protect other workers using the incubator.

Three people were actively engaged in the work, while 5 other individuals were employed in different capacities in the laboratory. Furthermore, our laboratory and animal house are the most centrally located ones in the Institute. The measures we used prevented a general outbreak of psittacosis, but, for some reason not known to us, they were insufficient to protect one of the doctors studying the disease. He came down with it. We believe that the infection took place by way of the upper respiratory tract, the portal of entry most difficult to protect.

EXPERIMENTAL

In the experimental work concerning psittacosis in parrots our chief interests were centered around the portal of entry of the virus, the distribution of the incitant in the body, and the portal of exit of the active agent. Information regarding these matters seemed essential for an understanding of the spread of the disease from bird to bird, and from birds to man. Incidentally, we were anxious to learn something of the clinical and pathological manifestations of the disease in parrots.

From examination of Text-fig. 1, one learns that Krumwiede demonstrated that emulsions of mixed organs or filtrates of these emulsions administered intraorally or intramuscularly were capable of infecting birds. Furthermore, he showed that an emulsion of spleen alone injected intramuscularly was infectious. It remained for us to determine whether the virus is in the blood, in the liver, in the nasal secretions, and in the feces.* For this purpose, the following experiments were performed.

Parrot N received intramuscularly 1 cc. of a 10 per cent spleen emulsion from Parrot M, Feb. 22, 1930. Transferred to Rockefeller Institute, Mar. 3, at which time it was sick. Mar. 5, stools loose, feathers ruffled. Mar. 6, died and was autopsied immediately. Lungs negative; purulent (sterile) pericarditis; liver enlarged and friable; spleen large and soft; intestines a dusky reddish color.

Parrot N₁ received, Mar. 8, intramuscularly and intraorally 3 cc. and 1.5 cc. respectively of a 10 per cent liver emulsion from Parrot N. Mar. 10, feathers rough, stools loose. Mar. 20, feathers rough, stools less watery, thin frothy discharge from nose. Mar. 26, bird seems well again. May 27, reinoculated intranasally with stool from Parrot 106, and found to be immune.

Parrot K, Feb. 15, 1930, inoculated intraorally and intramuscularly with organ filtrate from Parrot F. Feb. 17, sick. Feb. 21, transferred to Rockefeller Institute, feathers ruffled and stools loose. Chloroformed and autopsied immediately. 2 cc. of blood drawn from the heart: half cultured and found free from ordinary bacteria, half used for inoculation of Parrot 83. Lungs and heart appear normal. Liver shows on the surface several yellowish white lesions. Spleen enlarged; intestines a dusky reddish color. Cultures of spleen, liver, and heart muscle remained sterile.

Parrot 84, Feb. 21, received intramuscularly 2 cc. of a 10 per cent liver, spleen, and heart emulsion from Parrot K. Feb. 25, looks sick, eats poorly. Feb. 28, weak, falls from perch. Mar. 15, seems much better. April 3, very sick again. April 6, very thin and weak, stools loose. Died 12 noon and was autopsied immediately. Lungs and heart normal in appearance. Liver very large; over its surface and throughout the organ numerous whitish yellow areas noted. Liver not very friable and seems to have more connective tissue than normal. Spleen very large (tenfold increase in size) and soft. Cultures of liver, spleen, and lung remained sterile.

* We knew that the virus had been demonstrated in the droppings from infected parrots (Armstrong, C., McCoy, G. W., and Branham, S. E., *Pub. Health Rep., U. S. P. H.*, 1930, 45, 725). The fact, however, that the inciting agent is found in droppings collected from the floor of cages is not definite evidence that the virus is excreted in the feces, because the droppings might become contaminated with virus after passage from the body.

Parrot 83, Feb. 21, inoculated intramuscularly with 1 cc. of blood from Parrot K. *Feb. 26*, seems slightly ill, feathers somewhat ruffled. *Mar. 10*, bird died rather unexpectedly, inasmuch as it had never seemed very sick. Autopsy showed normal lungs, liver, and brain, purulent (sterile) pericarditis, and friable spleen. Cultures of heart muscle, liver, and spleen remained sterile.

Parrot 86, Mar. 12, received 2.5 cc. and 0.5 cc. of an emulsion of liver and spleen intramuscularly and intraorally respectively. Remained well until *Mar. 20*, when for the first time it looked sick, feathers roughened, stools loose. *Mar. 23*, better, stools formed. *Mar. 29*, very sick again, weak, feathers rough, stools loose. *Mar. 30*, chloroformed and autopsied immediately. Emaciated, lungs normal, no pericarditis, liver large and friable, spleen twice normal size and soft, intestines injected, brain normal. Cultures of lungs, liver, and spleen remained sterile.

Parrot 91, Mar. 30, inoculated intramuscularly with 4 cc. of a 10 per cent liver and spleen emulsion from Parrot 86. Also fed 1 cc. of the emulsion. *Apr. 7*, sick for the first time. *Apr. 11*, sick, stools loose. *Apr. 18*, very sick, stools watery, stools collected for filtration experiment. *Apr. 19*, died and was autopsied immediately. Not emaciated. Lungs fairly normal, small hemorrhages in visceral pericardium. Liver large and friable; throughout the organ numerous yellowish white areas of varying size (Fig. 4). Spleen 3 or 4 times normal size and spotted (Fig. 3). Intestines injected. Cultures of lungs showed several kinds of bacteria. Cultures of liver and spleen remained sterile.

Parrot 95, Apr. 18, stool collected from Parrot 91 was diluted with Locke's solution and filtered (half-hour) through a Berkefeld V. It was again filtered (10 minutes) through another Berkefeld V. The filtrate was free from ordinary bacteria. Of this filtrate Parrot 95 received 2 cc. intramuscularly, 1 cc. intraorally, and 5 cc. in its drinking water. *Apr. 26*, stools loose for first time. *May 8*, bird has gradually become worse; weak, eats poorly, feathers roughened. *May 14*, died. Immediately after death, material was collected from nose. Autopsy: Lungs normal; heart covered with a flaky exudate; liver large, friable, and mottled with yellowish areas of varying size; spleen 3 times normal size; flaky exudate over spleen and liver. Cultures of liver, spleen, and exudate remained sterile.

Parrot 96, Apr. 19, received intraorally and intranasally 1 cc. of a 10 per cent liver and spleen emulsion from Parrot 91. 3 cc. of the emulsion was also put on the food and in the drinking water. *Apr. 24*, stools loose. *Apr. 29*, bird very sick and weak, stools watery. *Apr. 30*, bird worse, chloroformed. After death a whitish material came from nose (probably from procrop also). The bird was held over a Petri dish into which the material was allowed to drop. Autopsy: Lungs normal; no pericarditis; liver fatty and friable, studded with numerous white spots; spleen 5 times normal size, pale and friable; intestines normal; brain injected. Cultures of liver and spleen remained sterile. Spleen was contaminated while being removed. Cultures of nasal secretions showed no non-lactose-fermenting bacilli.

Parrot 98, Apr. 30, 0.25 cc. of the nasal secretions collected from Parrot 96 were

dropped into the nose of Parrot 98. *May 2*, stools loose, but bird seems to be in fairly good condition. *May 5*, bird died rather unexpectedly. *Autopsy*: Hemorrhages in pericardium; lungs normal; liver large, fatty, friable, and studded with whitish spots; spleen 5 times the normal size and friable. Increase in the amount of peritoneal fluid. Cultures of this fluid remained sterile. Anaerobic and aerobic cultures of the spleen and the aerobic cultures of the liver showed no bacteria. From the anaerobic cultures of the liver a small Gram-negative influenza-like bacillus, that grew on blood agar and not on plain agar, was obtained.

Parrot 105, May 14, Parrot 95, immediately after death, was suspended, head down, over a Petri dish. 0.25 cc. of a thick whitish material were collected in this manner, and, having been diluted with a small amount of Locke's solution, were instilled in the nose and mouth of Parrot 105. *May 16*, stools slightly loose. *May 22*, stools watery, bird quiet, feathers roughened. *July 7*, the bird has been having recurring attacks of diarrhea. In spite of these attacks the parrot seems in good condition. Chloroformed and immediately autopsied. Not emaciated. Lungs and heart normal. Liver is extraordinary: the organ is adherent to surrounding structures, left lobe reduced to an unrecognizable small mass of scar tissue; right lobe about one-half normal size, mottled, rubbery, sectioned with difficulty. An a-p section through the whole lobe shows scar tissue in the center with normal liver tissue posteriorly. The appearance of the tissues suggests a healing or a chronic psittacosis infection. Spleen small with a whitish thickened capsule. Intestines adherent to each other in a manner suggesting a healing peritonitis. Duodenum red and inflamed. Cultures of liver and spleen remained free from ordinary bacteria.

Parrot 106, May 14, after Parrot 95 was dead and when its feathers had been plucked, the bird was dipped in 5 per cent lysol. Then the lysol was washed off with alcohol. By means of a sterile catheter attached to a syringe, 5 cc. of Locke's solution were injected into the cloaca and then withdrawn again. In this manner, fecal material was collected free from outside contaminants. 1 cc. of this material was instilled in the nose of Parrot 106. *May 19*, stools loose. *May 22*, bird worse, feathers roughened. *May 25*, bird very weak, right eye closed, stools watery. *May 26*, found dead. *Autopsy*: Bird emaciated. Pericardium, heart, and lungs normal. Liver large, fatty, friable; along interlobar fissure are yellowish areas of necrosis surrounded by bright red hemorrhagic zones, numerous similar but smaller areas ranging from pin points to peas in size were scattered through the organ. Spleen 4 times normal size and friable. Cultures of liver revealed no ordinary bacteria.

Parrot 107, May 28, stool collected from Parrot 106 in a manner similar to that employed with Parrot 95 (see Parrot 106 for details). 1 cc. of this fecal material was instilled in the nose and mouth of Parrot 107. *May 30*, stools loose. *June 2*, bird worse. *June 11*, the bird has been gradually getting worse; weak, feathers roughened, severe diarrhea. Chloroformed and autopsied immediately. Lungs normal. Pericardium contains 0.5 cc. of purulent-looking, sticky exudate. Liver

smaller than normal and shows numerous necrotic zones. Spleen 3 times normal size and friable. Cultures of liver remained sterile. Smears from liver and spleen showed no "minute bodies." Preparations from the pericardial exudate stained according to a modification of Castaneda's methylene blue safranin method* revealed numerous "minute bodies," of the type first described by Levinthal (6).

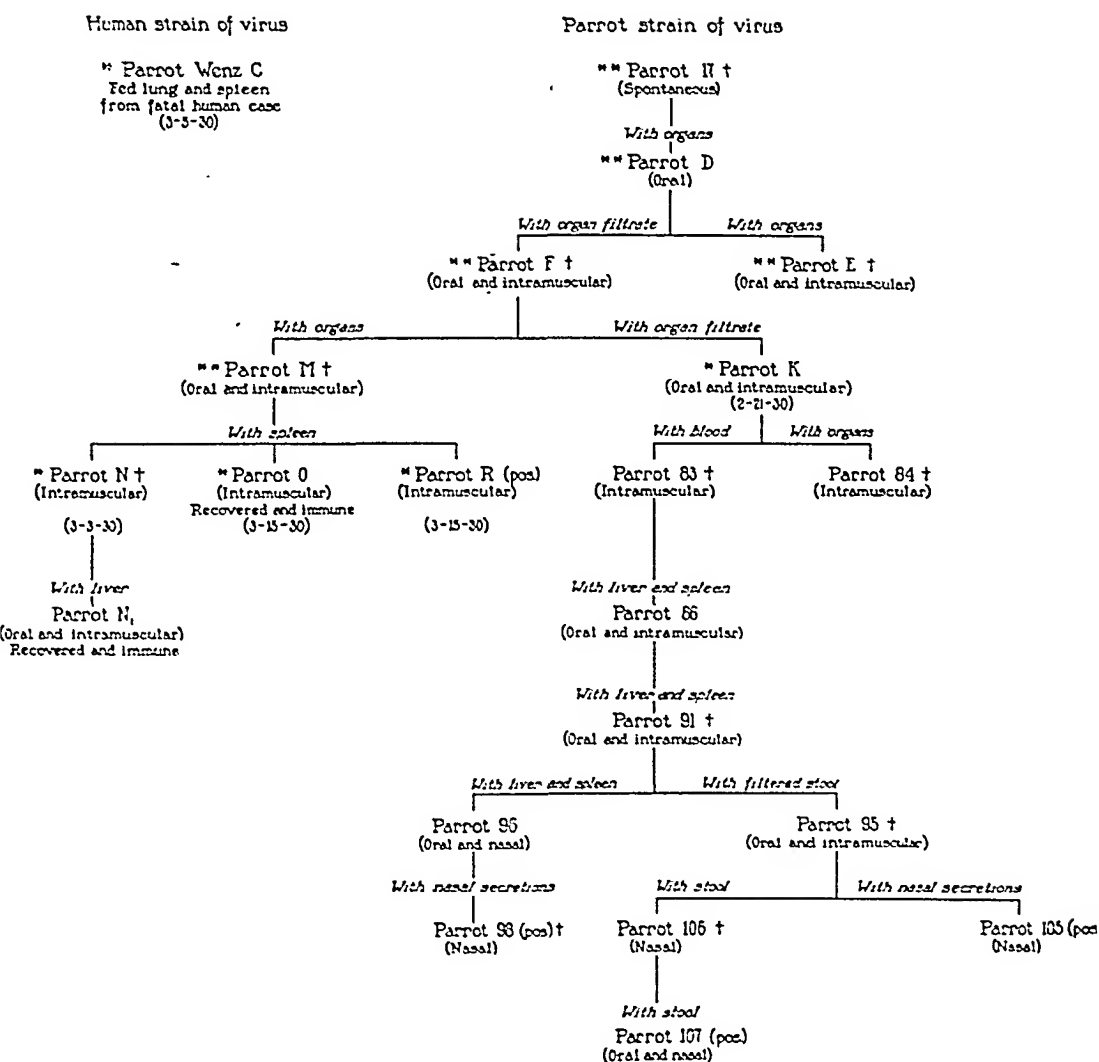
The experiments detailed above are summarized in Text-fig. 1. From an examination of the results, certain facts become obvious. In the first place, the virus of psittacosis is found in the stools, in a mixture of nasal secretions and material from the procrop, in the blood, in the liver, and in the spleen of infected birds. Moreover, parrots are capable of being infected by intramuscular, intranasal, or intraoral inoculations of the virus. These facts indicate the manner in which the disease spreads from bird to bird, and also suggest the source of infection for man.

The clinical picture of psittacosis in birds varies. The disease may be acute, running its course in a few days, or it may be chronic, enduring for several months. The birds may die suddenly without showing appreciable signs of illness. As a rule, however, loss of weight, roughening of the feathers, weakness, watery stools, and discharge from the nose are observed during the course of the disease. In our experience, the majority of the infected birds died. The gross pathology is characterized by an occasional sterile pericarditis, enlarged fatty livers, many of which show areas of necrosis (Fig. 4) or infarction, enlarged friable spleens (Fig. 3), and injected intestines. Regardless of the mode of inoculation, none of the parrots revealed changes in the lungs that might be attributed to psittacosis.

Microscopic Pathology

Spleen.—Changes in the lymphoid follicles vary from slight alterations to an almost complete obliteration of their normal architecture (Figs. 5 and 6), while

* Phosphate buffer pH 7.0.....	95
Formalin.....	5
Loeffler's methylene blue.....	10
Stain 2 minutes, rinse with tap water, and quickly counterstain with aqueous safranin.....	



TEXT-FIG. 1. Diagrammatic representation of the experimental work concerning psittacosis conducted in parrots. The doubly starred birds were handled only by Krumwiede, the singly starred parrots were handled both by Krumwiede and by us, the birds without stars were handled only by us. † indicates that the bird died.

the reticular and sinus structures are well preserved. The organ is infiltrated with wandering phagocytic cells with vacuolated cytoplasm containing amorphous debris, pigment, and globules of fat. The increase in the size of the spleen seems to be due to the content of blood and the enormous number of mononuclear cells.

*Liver.**—The characteristic lesion of psittacosis in parrots consists of multiple discrete areas of necrotic liver cells irregularly distributed throughout the organ, but with a tendency to be more numerous near its periphery. The condition appears to have its onset in the death of isolated liver cells or groups of cells; the cytoplasm becomes acidophilic and granular, and shrinks from contact with other cells. The nuclei become hyperchromatic and pyknotic, and eventually disappear completely. At this stage, mononuclear phagocytes and a few polymorphonuclear cells surround and infiltrate the lesion. As the process progresses, the necrotic liver cells disintegrate, leaving strands of acidophilic, hyaline material which may or may not show collections of leucocytes and depositions of fibrin (Fig. 7). When necrotic areas penetrate to the surface of the liver, accumulations of inflammatory cells are seen under Glisson's capsule, and an extension of the process leads to the perihepatitis and peritonitis that are encountered. Around the zones of necrosis, proliferation of liver cells, indicated by mitotic figures, occurs. Within and around the lesions, cells, for the most part "endothelial leucocytes," filled with the "minute psittacosis bodies," are also found (Fig. 2).

Throughout the liver there is a proliferation of Kupffer cells and focal accumulations of wandering mononuclear cells of the same general type. Some of these have a highly vacuolated cytoplasm containing masses of fat. Many plasma cells are also present.

The bile ducts do not escape injury. Within the necrotic areas, they are dilated, and at times contain numerous mononuclear phagocytes. The cells that form the walls of the ducts may undergo necrosis, becoming granular with pyknotic or missing nuclei. In chronic and in healing lesions, irregularly shaped collections of hepatic cells separated from each other by various sized bile ducts undergoing proliferation and surrounded by lymphocytes and fibrous tissue (Fig. 8) are observed. The dilatation of the bile ducts and the character of the lesions in the later stages of the disease which resemble those produced by ligation of the common duct, lead one to believe that occlusions of the biliary system in the parrot may occur in some manner as the result of an infection with the virus of psittacosis.

Striking alterations in the vascular channels are not usually seen, but in certain

* The anatomy of the parrot's liver differs from that of mammalian livers in that the bile duct draining the left lobe enters directly into the duodenum. There are also differences in the microscopic appearances of the two kinds of livers. For example, lobulation in the parrot's liver is not well developed and arrangement of the parenchymal cells in chains occurs only to a slight extent. Moreover, the bile ducts, which in mammals are invariably associated with blood vessels, may at times be found unassociated with such structures in the parrot.

TABLE I
Summary of Reinoculation Experiments in Parrots

Parrot No.	1st inoculation Material Route, date	Virus in inoculum*	Course after inoculation	Time between inoculations	Reinoculation Material Route, date	Course after inoculation	Autopsy	Active immunity + or -
O	Parrot spleen i.m. 2-22-30	+	Sick 1 mo.	days 95	All inoculated in manner identical with control Parrot 107 5-28-30	Negative	Lived	+
McG	Human blood i.m. 3-7-30	-	Negative	82		Died on 9th day	Psittacosis	-
N ₁	Parrot liver Oral and i.m. 3-8-30	+	Sick 3 wks.	81		Occasional mild diarrhea	Lived	+
89	Mouse organs Oral and i.m. 3-28-30	+	Sick 1 mo.	61		Negative	Lived	+
92	Human blood Oral, i.p., i.m. 4-3-30	-	Negative	55		Typical illness, killed after 9 days	Psittacosis	-
93	Human nasal washings Oral 4-3-30	?	Very slight diarrhea	55		Typical illness, killed after 6 days	Psittacosis	-
107	Control—Inoculated by oral and nasal instillation of 1 cc. of stool from Parrot 106, sick with psittacosis. Was sick 48 hrs. after inoculation; ran fulminating course; was killed when moribund on 14th day. Autopsy showed typical psittacosis							
102	Human lung Oral and i.m. 5-9-30	-	Negative	31	Mouse organs Oral and i.m. 6-9-30	Typical illness, killed on 8th day	Psittacosis	-

Control—Material used for reinoculation of Parrot 102 produced psittacosis in Mice WC₂₄ and Monkeys H, I, and J.

* Presence or absence of virus in inoculum determined in addition by other animal inoculations.

cases fibrin thrombi, involving portal vessels, are found. In most instances, only small vessels are occluded, but occasionally a large branch is involved. The question as to whether the areas of necrosis result from vascular thromboses or whether the thromboses are caused by necrosis cannot be definitely answered. But the evidence is such that it seems unlikely that much of the necrosis is the result of vascular occlusions.

Immunity

Having investigated the portal of entry and exit of the virus in parrots and upon concluding the study of the clinical and pathological pictures of the disease in its natural host, we then became interested in determining whether the birds that had recovered from psittacosis were resistant to reinfection.

For the work on active immunity, 7 parrots were available; 3 had recovered from psittacosis experimentally induced 61-91 days prior to the reinoculation, 3 had been inoculated 31-82 days previously with material subsequently shown to be free of virus, 1 had received intraorally either a very small amount of virus or none at all. In any event, the last bird mentioned evidenced few if any signs of illness following the first inoculation. 6 of the birds (O, McG, N, 89, 92, 93) and a control received intranasally and intraorally 1 cc. each of an unfiltered stool from Parrot 106 sick with psittacosis. The seventh parrot in the group (102) was tested for immunity by means of a virus-containing emulsion from mice. The 3 parrots (O, N, 89) that had recovered from a previous infection lived, while the others developed psittacosis and reacted in a manner similar to that of the control. The results of these experiments are summarized in Table I.

From the experiments described above and summarized in Table I, it is obvious that parrots are actively immune following an attack of psittacosis.

DISCUSSION

The results of our investigations concerning psittacosis experimentally induced in parrots need few comments. It seems advisable, however, to emphasize again the danger of studying the disease in parrots. Due to the presence of virus in the nasal secretions and feces of infected birds and because of the parrot's filthy habits, to protect oneself against the entry of dried virus into the upper respiratory tract is extremely difficult. In view of this fact, and since mice are suitable for diagnostic and experimental work, as demonstrated in the second paper of this series, investigations with parrots should be limited as much as possible.

The "minute bodies" found in exudates and in certain infected organs were first described by Levinthal (6) who thinks that they are of etiological significance and probably represent small bacteria of a nature similar to that of *B. tularensis*. Lillie (7) believes these structures are *Rickettsiae* and proposes for them the name *Rickettsia psittaci*. Coles (8) speaks of them as "x-bodies" and is also of the opinion that they probably constitute the causal agent. We have experienced no difficulty in finding these bodies in some animals, while in others extensive search has failed to reveal them. When present they take stains with ease, are Gram-negative, and closely resemble minute microorganisms with a diameter of about 0.2μ . As yet, however, no one has succeeded in cultivating them on ordinary laboratory media, and their exact nature and relation to psittacosis is still an open question.

CONCLUSIONS

1. The virus of psittacosis is present in the nasal secretions, feces, blood, spleen, and liver of an infected parrot.
2. Parrots are susceptible to intraoral, intranasal, or intramuscular inoculations of the virus.
3. The most constant pathological changes produced by psittacosis in parrots occur in the spleen and liver. The lesions exhibited in the latter organ consist of areas of necrotic liver cells and damage to bile ducts. In no instance, in our experience, were lesions observed in a parrot's lungs comparable to those found in the lungs of men.
4. "Minute bodies" similar to those described by Levinthal and others were found in many, but not in all of the infected birds.
5. Parrots that have recovered from one attack of psittacosis exhibit an active immunity against reinfection.

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EXPLANATION OF PLATES

PLATE 5

FIG. 1. Photograph of costume worn in animal rooms. A parrot with psittacosis is perched on the left wrist of the worker. The other bird is normal.

FIG. 2. "Minute bodies" in mononuclear cells of the liver. $\times 1700$.

FIG. 3. An enlarged and mottled spleen from a parrot with psittacosis. $\times 1$.

FIG. 4. Liver from a parrot infected with psittacosis. The necrotic areas are white. $\times 1$.

PLATE 6

FIG. 5. Section from a parrot's spleen injured by the virus of psittacosis. Normal architecture destroyed. $\times 170$. Eosin and methylene blue.

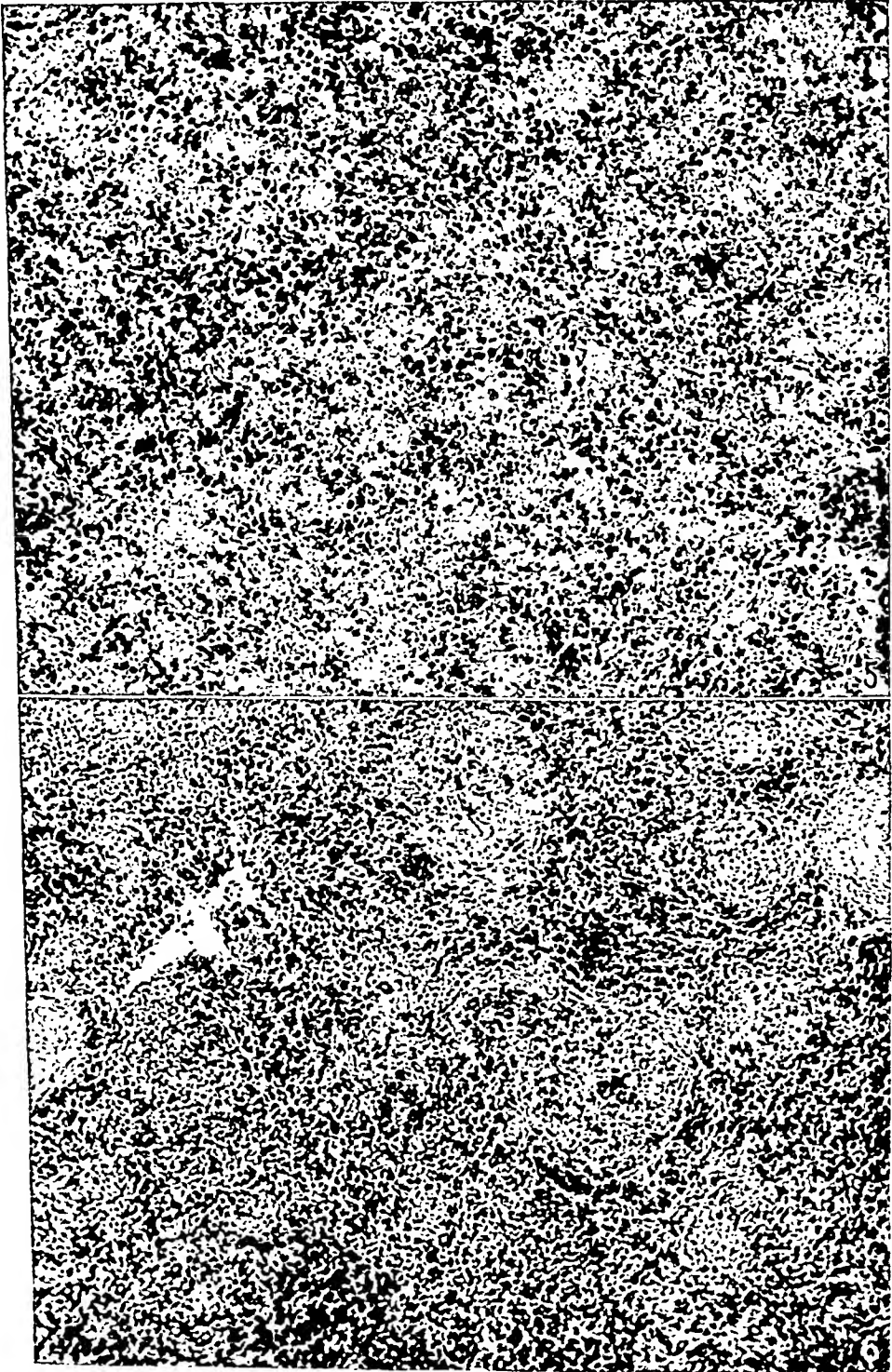
FIG. 6. Normal parrot's spleen. Compare with Fig. 5. $\times 170$. Eosin and methylene blue.

PLATE 7

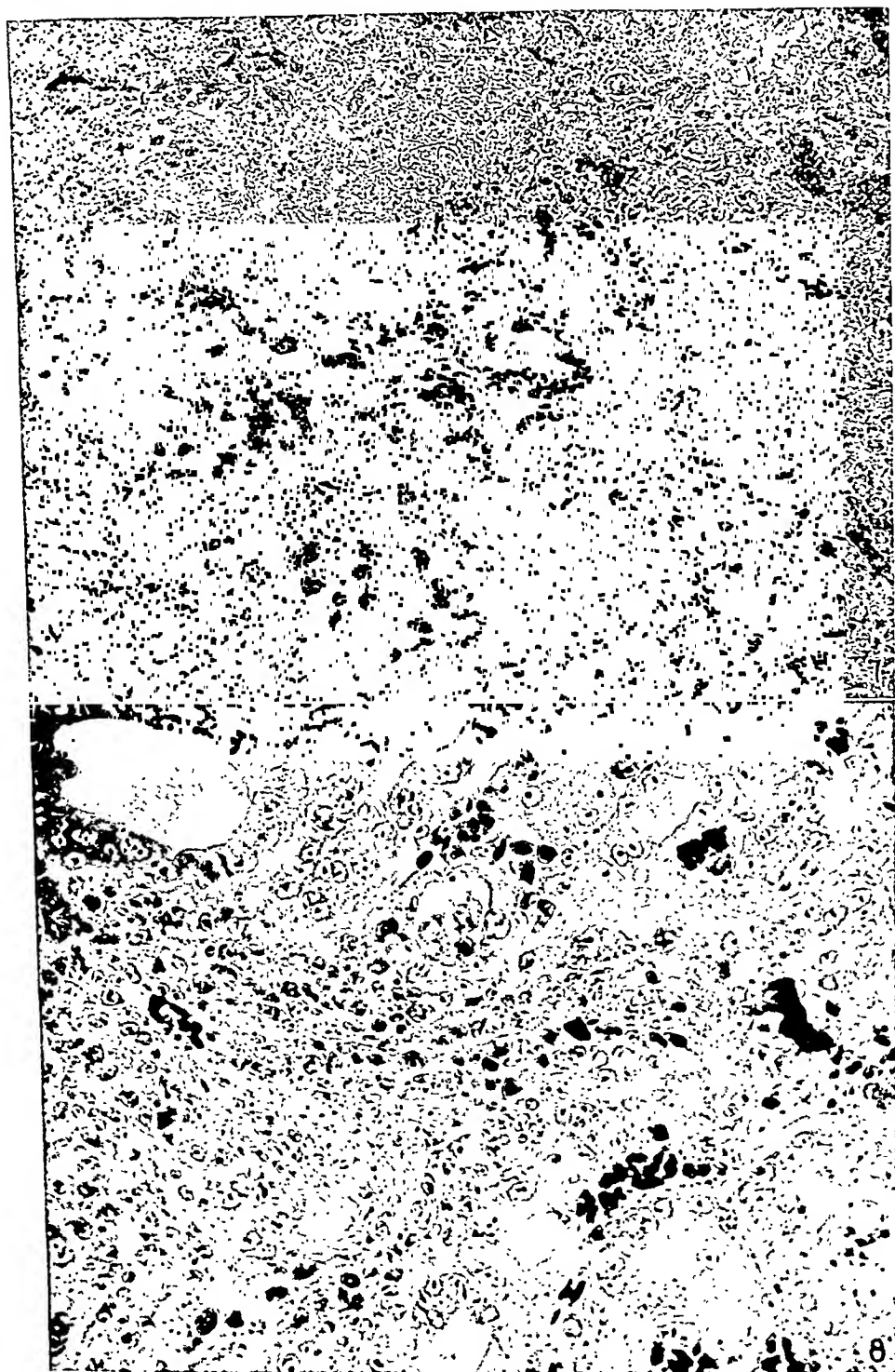
FIG. 7. Section from a liver of a parrot with psittacosis. Early lesions showing degeneration of liver cells and depositions of fibrin. $\times 115$. Eosin and methylene blue.

FIG. 8. Section from a liver late in the disease, showing proliferation of bile ducts, infiltration of mononuclear cells, and deposition of connective tissue. $\times 450$. Eosin and methylene blue.





Photographed by Louis Schmidt



Photographed by Louis Schmidt

(Rivers *et al.*: Psittacosis. D)

PSITTACOSIS

II. EXPERIMENTALLY INDUCED INFECTIONS IN MICE

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PLATES 8 TO 10

(Received for publication, April 1, 1931)

Krumwiede and his coworkers (1) were the first to report that mice are capable of being experimentally infected by intraperitoneal inoculations of the virus of psittacosis derived from a parrot. Furthermore, they showed that the active agent can be transmitted from mouse to mouse in series. These observations were important because they provided a readily available experimental animal and one much less dangerous to handle than are parrots. Consequently, when Krumwiede and his associates discontinued their work and gave us two strains of virus, observations on psittacosis infections in mice were immediately begun. The object of this communication is to describe some of our experiences encountered during the course of these investigations.

Methods and Materials

Virus.—Active agents from 3 sources were used. One strain (Parrot N) came from a bird (see first paper in this series) infected with virus originally obtained from a sick parrot. The second strain (Wenz C or WC) was secured from a parrot inoculated with material from a patient who had died of psittacosis. The third virus was obtained from the sputum of a patient (G. P. B.) during the course of a psittacosis infection contracted in the laboratory (2). From 2 sources in the mice, *viz.*, brain, pooled liver and spleen, the virus was secured for passages and experimental purposes. The organs used were always tested for the presence of ordinary aerobic and anaerobic bacteria.

Inoculation.—The mice were usually inoculated intraperitoneally or intracerebrally with 0.5 cc. and 0.025 cc. respectively of organ emulsions. The technique of intracerebral injections will be described in detail later in the paper.

Clinical Signs of Infection in Mice.—In animals inoculated intraperitoneally, the time of onset of the illness varied with the amount and potency of the virus.

Loss of appetite and weight, and roughening of the fur were constant signs of infection. A few animals died during convulsive seizures. The elapse of time between the inoculations and the death of the animals varied, *e.g.*, many mice died within 48 hours, others succumbed after 3 weeks, and a few recovered. The clinical picture of the disease in mice intracerebrally inoculated will be discussed in another part of the paper.

EXPERIMENTAL

Three strains of psittacosis virus were studied experimentally in mice. A brief review of the findings with 2 of them will be made, after which follows a more detailed description of the work with the third. Then, the pathological changes caused by the virus in mice will be discussed. Finally, certain experiments dealing with the phenomena of immunity will be described.

Mice Infected with a Parrot Strain of Virus

The following experiments were performed to ascertain whether a parrot strain of psittacosis virus is capable of propagation in mice.

A 10 per cent emulsion of liver and spleen from Parrot N was divided into 2 portions, one of which was heated at 100°C. for 1 minute. Then 4 mice were inoculated intraperitoneally (0.5 cc. each) with the heated, and 4 more with the unheated material. None of the animals that received the heated emulsion became ill, while all of those that were inoculated with the unheated material sickened and died. An emulsion was made from the liver and spleen of one of the mice that died. Again the emulsion was divided and treated in a manner similar to that already described. The 4 mice inoculated with the heated material remained healthy, and later, when 2 of them had been sacrificed, 4 other mice were injected with an emulsion of their livers and spleens. These animals showed no illness. The mice, however, that received the unheated emulsion died in 4-7 days. By means of intraperitoneal inoculations of liver and spleen emulsions, serial passages of the virus through 7 successive groups of mice were made, and the typical clinical and pathological pictures of psittacosis were observed in the infected animals. The series was discontinued after the 7th passage.

The above group of experiments shows that a parrot strain of psittacosis virus is pathogenic for mice, and that the virus can be propagated serially in mice by means of intraperitoneal inoculations of infected hepatic and splenic emulsions. Moreover, virus-containing emulsions that have been heated at 100°C. for 1 minute and emulsions of livers and spleens from normal mice are innocuous.

Mice Infected with B-Sputum Strain of Virus

An infection incurred in the laboratory provided an opportunity of determining (1) whether psittacosis virus occurs in a patient's sputum, and (2) whether mice are suitable for diagnostic purposes.

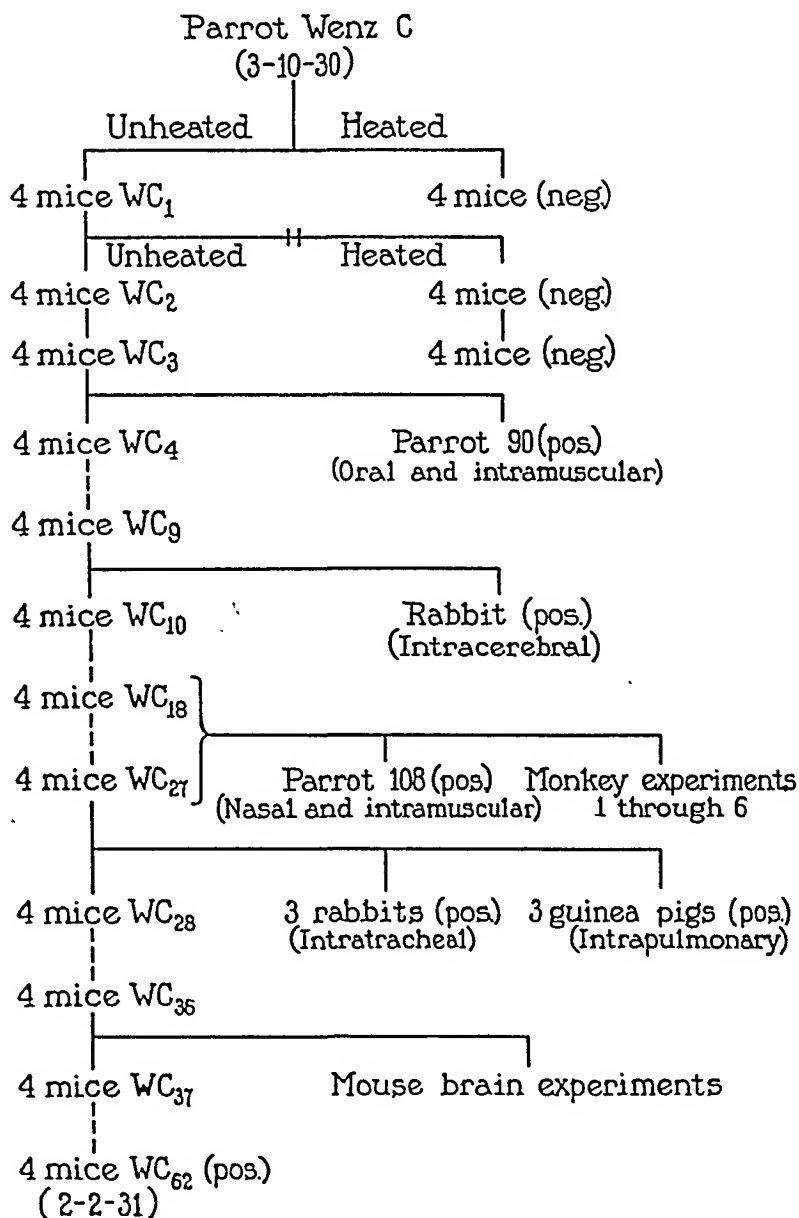
G. P. B. expectorated a mass of thick tenacious sputum on the 10th day of his illness (2). This sputum was washed and emulsified in Locke's solution. Cultures of the emulsion showed a few colonies of organisms usually found in the throat. Apr. 10, each of 4 mice received intraperitoneally 0.5 cc. of the emulsified sputum. On Apr. 12th, one mouse was found dead. Since it had evidently died of a bacterial infection, no passage from it was made. On Apr. 25th, however, another mouse died, and shortly after death an emulsion of its liver and spleen, which was free from ordinary bacteria, was injected into normal mice. These animals sickened and died in 3 or 4 days. With liver and spleen emulsions passages were made in series through 13 sets of mice at which time they were discontinued. The majority of the animals died 3-7 days after inoculation and presented a typical picture of psittacosis. Some of the material used for the 4th transfer induced psittacosis when injected into Parrot 102.

The above experiments clearly indicate that mice may be used for the diagnosis of psittacosis. Furthermore, they demonstrated for the first time—and the results were confirmed by inoculation of a parrot—that the virus is present in the sputum of man infected with psittacosis. Bedson and Western (3) state that Krumwiede (1) proved the presence of virus in a patient's sputum. But the report of Krumwiede and his coworkers shows that they demonstrated the active agent in a mixture of sputum and blood. Since it is known that the virus is frequently in the blood, such an experiment does not suffice to prove its presence in the sputum. Nevertheless, it is the virus in the sputum which probably constitutes the chief source of danger in handling patients with psittacosis.*

Mice Infected with Wenz C Strain of Virus

Parrot Wenz C was inoculated by Krumwiede with an emulsion of lung and spleen from a woman who had succumbed to psittacosis.

* A small epidemic of psittacosis recently occurred in Brooklyn. Mice alone were used to obtain the virus from sputum, and autopsy material. They were adequate for diagnostic purposes. The details of this work will be published later.



TEXT-FIG. 1. Schematic portrayal of the experimental work in mice dealing with the WC strain of psittacosis virus.

Shortly after inoculation the bird was transferred to The Rockefeller Institute, where it soon became sick. From a sterile emulsion of the liver and spleen of this parrot a series of passages and experiments

in mice, schematically portrayed in Text-figs. 1 and 2, were initiated March 10, 1930.

Serial Passages by Intraperitoneal Injections of Emulsified Livers and Spleens.—In Text-fig. 1 the course of the work is graphically depicted: the mode of transfer, when not designated, consisted of an intraperitoneal injection of an emulsion of pooled liver and spleen. From the diagrammatic representation of the experiments, one ascertainment that serial passage of the virus has been made on 62 occasions extending over a period of nearly a year. Moreover, it is obvious that heated emulsions of livers and spleens from infected parrots and mice and unheated emulsions of livers and spleens of normal mice did not induce psittacosis. Furthermore, it was demonstrated that the virus propagated in mice is pathogenic for parrots, rabbits, guinea pigs, and monkeys.

Passage of the virus through mice in some manner caused it to become more potent for this host.

Early in the work, 0.5 cc. of a 10 per cent organ emulsion required 4 or 5 days to kill the animals, and a few mice survived. After the 40th passage, however, such a dose was regularly fatal within 48 hours. Titrations of the virus after 60 passages gave the following results: 4 mice were inoculated with 0.5 cc. of each dilution—10 per cent, all dead in 48 hours; 1 per cent, all dead in 3–5 days; 0.1 per cent, all dead in 6 days; 0.01 per cent, all dead in 8–9 days; 0.001 per cent, all dead in 8–9 days; 0.0001 per cent, all dead in 10–11 days. Thus, it appears that the virus is now quite virulent for mice, and there is no reason to suppose that it will not retain its potency.

On the basis of the work just presented, one is justified in concluding that at least certain strains of psittacosis virus are capable of being propagated indefinitely from mouse to mouse by intraperitoneal injections of emulsified livers and spleens. Our own work with rabbits and guinea pigs, to be reported in the third communication of this series, and the experiences of different investigators in producing herpetic infections (4) and yellow fever (5) in mice by intracranial inoculations of infectious material induced us to attempt serial passages of psittacosis virus in the brains of these animals.

Intracerebral Inoculations

Technique.—Intracerebral inoculations* in mice are accomplished with ease. A 0.25 cc. tuberculin syringe fitted with a 27 gauge needle, was used. With the body of the mouse under the palm of the left hand, one grasps the head between the thumb and index finger and presses it firmly against the table. The fur is then parted over the middle of the left parietal bone by means of a small cotton swab moistened with 95 per cent alcohol. The right hand holding the syringe slowly pushes the needle through the skull, 2 mm. to the left of the midline, to a depth of about 2 mm. and injects 0.025 cc. of the inoculum. If serum, Locke's solution, or normal brain emulsion is injected in this manner, the mice appear normal within 30–60 minutes. If irritating fluids are introduced, 24 hours may be required for the return to normal.

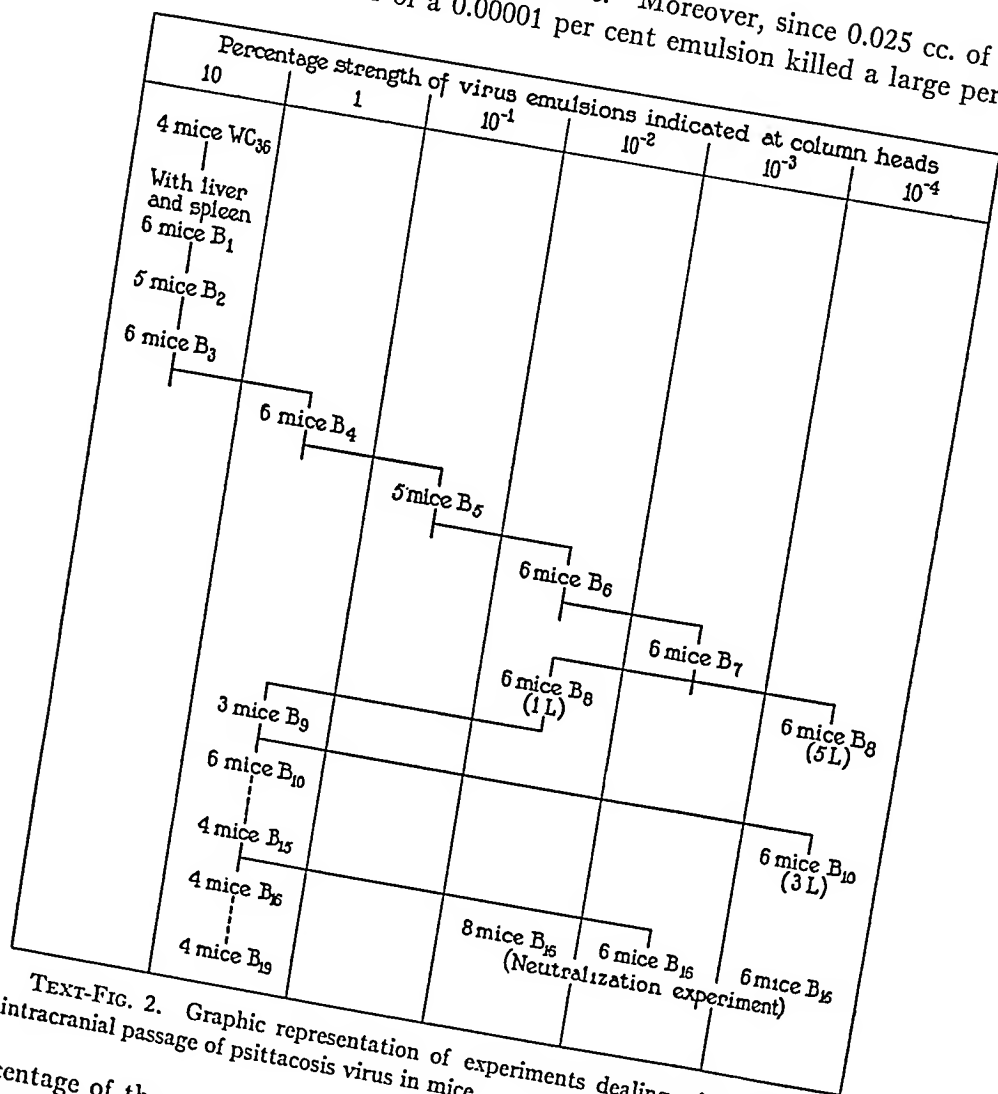
Clinical Picture and Time of Death.—Except for the mice that received the most concentrated emulsions, the animals appeared normal the day following inoculation and so remained from one to several days according to the dose. Death usually occurred within 48 hours after the appearance of signs of illness. Failure to eat and hyperirritability accompanied by roughening of the fur were the first evidences of sickness. The irritability manifested itself in exaggerated responses to different stimuli. Between the periods of motor hyperactivity, the animals sat quietly with arched backs and with heads held perpendicular to the floor. At this stage of the disease, the eyes were surrounded by red circles. Twitchings involving the jaw, eyes, and legs developed. Ataxia was pronounced and some of the mice went round in circles, or would rise up and fall over backwards. Then, generalized convulsive seizures developed and death followed. The posture in death was characteristic and striking; the head was retracted, the back was arched, the forelegs were flexed, the hindlegs and tail were extended. Immediately after death, rigor mortis developed. The time of death varied with the dilution of the inoculum. The following results illustrate this fact: 4 mice were inoculated with 0.025 cc. of each dilution—1 per cent, all dead in 40–60 hours; 0.1 per cent, all dead 3–4 days; 0.01 per cent, all dead 3–4 days; 0.001 per cent, all dead 4–5 days; 0.0001 per cent, all dead 5–7 days; 0.00001 per cent, all dead 6–7 days. The potency of emulsions varies from time to time, and some of the mice that received the higher dilutions occasionally survived.

Results

The passages of virus from brain to brain were initiated by the intracerebral inoculation of 6 mice with 0.025 cc. each of a 10 per cent emulsion of livers and spleens from mice WC₃₆ (see Text-figs. 1 and 2). The results of the work portrayed in Text-fig. 2 demonstrate that the virus by intracranial inoculations of emulsified brain tissue was serially

* All operations were performed under light ether anesthesia.

propagated through 19 sets of mice. Moreover, since 0.025 cc. of a 0.0001 per cent or of a 0.00001 per cent emulsion killed a large per-



TEXT-FIG. 2. Graphic representation of experiments dealing with the serial intracranial passage of psittacosis virus in mice.

centage of the mice, it appears that the potency of the virus in the brain was equal to, if not greater than that in the livers and spleens.

Pathology

The pathological findings vary somewhat according to the mode of inoculation and the lapse of time between inoculation and death. The gross changes in the different sets of animals will be described first, after which the microscopic pathology will be discussed.

Gross Pathology of Mice Inoculated Intraperitoneally.—The mice that received intraperitoneal injections of virus-containing emulsions were emaciated and frequently had distended abdomens. On opening the abdominal cavity, one usually found an excess of sticky fluid containing flakes of fibrin. The livers were enlarged and friable; often they had undergone such extensive fatty degeneration that they had become chamois-colored; and scattered over the surface yellowish white spots, surrounded occasionally by hemorrhagic zones, were at times seen. Such spots, most frequently observed at or near the edges of the lobes, resembled areas of necrosis or minute infarcts (Fig. 5). If the mice died promptly, enlargement and fatty degeneration were the only gross evidences of involvement of the liver. The longer the animals survived after infection, the more likely were the necrotic areas in the liver to be found. The spleens were usually enlarged, very red, and friable. At times whitish spots could be seen shining through the capsule. The pancreas, adrenals, and intestines were not particularly abnormal. The kidneys showed a mild parenchymatous degeneration. When the thorax was opened, the pleura and heart were usually normal in appearance. The lungs were redder than normal, but pneumonia was seldom if ever noted. Aerobic and anaerobic cultures of the livers and spleens with a few exceptions remained sterile. When bacteria were present, they were found to be of various sorts and of no etiological significance.

Gross Pathology of Mice Inoculated Intracerebrally.—The chief difference between the mice inoculated intracerebrally and those injected intraperitoneally was the involvement of the brain. In opening the cranial cavity, one often saw a small hemorrhage beneath the scalp and a hemorrhagic spot in the left hemisphere of the brain where the injections were made. The sutures of the skull were wide and bulging, and when pieces of the bones were removed, the congested edematous brain herniated through the opening. Covering the surface of the brain and lying in spaces between the lobes was a sticky exudate. Cultures of this exudate and brain tissue remained sterile. The organs in the thorax were normal. The abdomen was not distended, but, in a certain number of the animals, gross changes were noted in the livers and spleens. The former were usually fatty, and about 20 per cent of them showed areas of necrosis. The latter were enlarged, and necrotic zones were seen in approximately 10 per cent of them.

Microscopic Pathology. Liver.—When the mice were inoculated intraperitoneally, an exudate, consisting of fibrin, polymorphonuclear and mononuclear cells, at times was seen over the surface of the liver. The pathological pictures

presented by stained sections of the liver varied with the duration of the disease. During the acute stage, a general infiltration with fine globules of fat and a granular degeneration of the liver cells were present (Fig. 12). Isolated groups of cells with no apparent relation to the structural landmarks of the organ, underwent an acidophilic degeneration and finally disappeared leaving a pink reticulated mass which rapidly became infiltrated with polymorphonuclear cells (Figs. 6-9). If the animals survived more than several days or if they were killed during the period of convalescence, the liver cells were almost normal and the polymorphonuclear infiltrations had been replaced by compact nests of mononuclear cells (Figs. 10, 11). The cause of the necrosis is not known. In addition to the necrotic lesions, the sinuses of the liver contained large numbers of mononuclear cells, many of which were apparently derived from Kupffer cells.

Animals inoculated intracerebrally rarely exhibited a peritoneal exudate, but in a general way showed a type of pathology of the liver similar to that seen in the intraperitoneally infected mice with the exception that necrosis was not so prominent a part of the picture—occurring in about 20 per cent of the cases. The areas of necrosis in many respects resemble those found in human beings with typhoid fever and in mice infected with mouse typhoid. There are certain definite differences, however. For example, in the livers infected with the virus of psittacosis vascular thromboses are not an obvious part of the picture, while in mouse typhoid they are prominent. Moreover, the livers from mice with psittacosis are free from ordinary bacteria while this is not true of livers infected with mouse typhoid.

Spleen.—Stained sections from spleens (Fig. 1) of normal mice showed large well defined lymphoid follicles sharply separated by splenic pulp. Preparations from spleens damaged by psittacosis, however, did not stain well. The lymphoid follicles were less definite in outline and contained many necrotic or hyaline cells both centrally and peripherally situated (Figs. 2, 3). In the pulp, particularly around the periphery of the organ, individual necrotic cells or groups of them, and wandering phagocytic cells filled with amorphous debris were present. In the follicles and also in the pulp, the areas containing the degenerated or necrotic cells were infiltrated with polymorphonuclear elements and resembled minute abscesses (Fig. 4). Cultures from these spleens, however, remained free from ordinary bacteria. About 10 per cent of the spleens from mice intracerebrally infected also revealed areas of necrosis similar to those just described.

Brain.—Brains from intraperitoneally infected mice appeared normal, while those from animals intracerebrally inoculated showed a meningo-encephalitis. The reaction, chiefly meningeal, was characterized by an extensive exudate composed of polymorphonuclear and mononuclear cells. The blood vessels extending into the brain were surrounded by cellular infiltrations.

"Minute Bodies."—The minute coccobacillary structures originally described by Levinthal (6) and later designated as *Rickettsia psittaci* by Lillie (7), and as

"x-bodies" by Coles (8) were found in sections* and smears† of exudates and organs from mice infected with psittacosis. They were abundant in the peritoneal and meningeal exudates and in the livers and spleens. Not always, however, were they found in material known to be infectious. Furthermore, livers and spleens from mice that died within 48 hours after infection showed either none or only a few of the bodies, while organs from animals that survived a number of days contained many such structures. These bodies were small cocci or short bacillary structures, $0.2-0.25\mu$ in diameter or in length respectively. They occurred singly or in clusters intracellularly or extracellularly. For the most part, they were seen in the cytoplasm of mononuclear cells which were greatly distended by them. The nature of the bodies is not definitely known, but they appear to be minute organisms, and the idea, held by certain workers, that they represent the etiological agent of psittacosis deserves serious consideration.

Immunity

It seemed advisable to determine whether mice that had recovered from psittacosis were actively immune. Furthermore, an effort was made to ascertain whether the mouse can be used for testing the presence of protective antibodies in the sera of human beings convalescing from psittacosis. In this way, it was hoped that an easy experimental method might be found for the diagnosis of certain obscure conditions in man, thought to be psittacosis.

Active Immunity.—53 mice that had survived their original inoculation for varying periods of time were reinfected, 42 intraperitoneally and 11 intracerebrally. Of the 53 animals, only 5 lived for as long as 2 weeks. The others sickened and died in a manner identical with that of the controls. The reinfesting dose was 0.5 cc. of a 10 per cent organ emulsion that was employed for regular passages.

Protection Experiments.—4 groups of experiments were performed, involving 16

* In sections the bodies are colored blue with eosin and methylene blue and with Giemsa's stain.

† For the study of smears, a modification of the stain used by Castaneda in Zinsser's laboratory for the demonstration of Mooser bodies (*Rickettsiae*) in Mexican typhus was used. This stain colors the minute bodies blue and the cells and albuminous material pink.

	cc.
Phosphate buffer pH 7.0	95
Formalin	5
Loeffler's methylene blue	10

Stain 2 minutes, rinse with water, counterstain for a few seconds with aqueous safranin.

separate tests and 7 controls. Each test was carried out in 4 mice. Consequently 92 mice were used. Convalescent sera,* collected from 10 different patients 6-8 weeks after subsidence of fever, were tested. Serum from a rabbit that had recovered from an intracerebral inoculation of virus was also used. Normal human serum, normal rabbit serum, and Locke's solution were employed as controls. Each mouse was given intraperitoneally 0.5 cc. of normal serum, convalescent serum, or Locke's solution from 4-24 hours before the intraperitoneal administration of 0.5 cc. of a virus-containing emulsion. The animals in the test group showed no evidence of having been protected, inasmuch as they died as promptly as did the controls.

Neutralization Experiments.—One neutralization experiment, involving 18 mice, divided into 3 groups of 6 each, was performed. The serum tested was collected from a patient (G. P. B.) 10 days after subsidence of fever. The controls were Locke's solution, and normal human serum pooled from 5 individuals. The test and control sera, and Locke's solution respectively were mixed with an equal volume of virus-containing brain emulsion diluted to a percentage strength of 10^{-2} , 10^{-3} , 10^{-4} . The mixtures were incubated at 37°C . for 1 hour and then chilled for 1 hour, after which 0.025 cc. of each were intracerebrally injected in mice. All the mice responded to the inoculations in a similar manner. Thus, from this experiment no evidence that convalescent human serum contains an appreciable amount of neutralizing substances for psittacosis virus was obtained.

From the results of our investigations in mice concerning immunity to psittacosis it appears that little if any active resistance develops in these animals following one attack of the disease, that convalescent human serum does not protect them against virus administered 4-24 hours later, and, finally, that convalescent human serum possesses relatively small amounts of neutralizing antibodies. Although our experiments seem to indicate that no active immunity develops in mice, the evidence is not conclusive, inasmuch as an overwhelming dose of virus was used for reinoculation. Further work, therefore, is being conducted to obtain more accurate information.

SUMMARY AND CONCLUSIONS

The work presented in this communication concerning psittacosis in mice confirms Krumwiede's observations that mice inoculated intraperitoneally with emulsified livers and spleens containing the virus develop the disease and that the malady can in this way be passed serially through a number of mice. Furthermore, it has been

* Supplied by Drs. Amoss, Krumwiede, and Wadsworth.

shown that mice are susceptible to the virus administered intracerebrally and that the active agent can be propagated indefinitely by means of brain to brain inoculations. Moreover, by the use of mice, the presence of the virus of psittacosis in the sputum of a patient with the disease has for the first time been demonstrated. It follows that the mouse is available for diagnostic purposes.

The pathological findings in infected mice consist of enlarged fatty livers that frequently show areas of necrosis infiltrated with polymorphonuclear and mononuclear cells; enlarged spleens with areas of necrosis and cellular infiltrations involving the pulp and lymphoid follicles; and, finally, in intracerebrally infected animals, a meningoencephalitis. The "minute bodies" described by other observers were not found in all animals, but they were seen with sufficient frequency in smears of peritoneal and meningeal exudates and in smears and sections of livers and spleens to demand serious consideration as the possible etiological agent of the disease.

Neutralizing and protective antibodies were not found in convalescent human sera when the mouse was used as the test animal.

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EXPLANATION OF PLATES

PLATE 8

FIG. 1. Section from spleen of a normal mouse. Compare with Fig. 2. $\times 85$. Eosin and methylene blue.

FIG. 2. Section from spleen of mouse with psittacosis. Lymphoid follicles and pulp extensively involved. Compare with Fig. 1. $\times 85$. Eosin and methylene blue.

FIG. 3. Small area of hyaline necrosis in lymphoid follicle of spleen. $\times 375$. Eosin and methylene blue.

FIG. 4. Lesion in splenic pulp; area of hyaline necrosis infiltrated with polymorphonuclear leucocytes. $\times 375$. Eosin and methylene blue.

PLATE 9

FIG. 5. Liver from mouse with psittacosis. The organ has undergone fatty degeneration, and is mottled with areas of focal necrosis. $\times 1$.

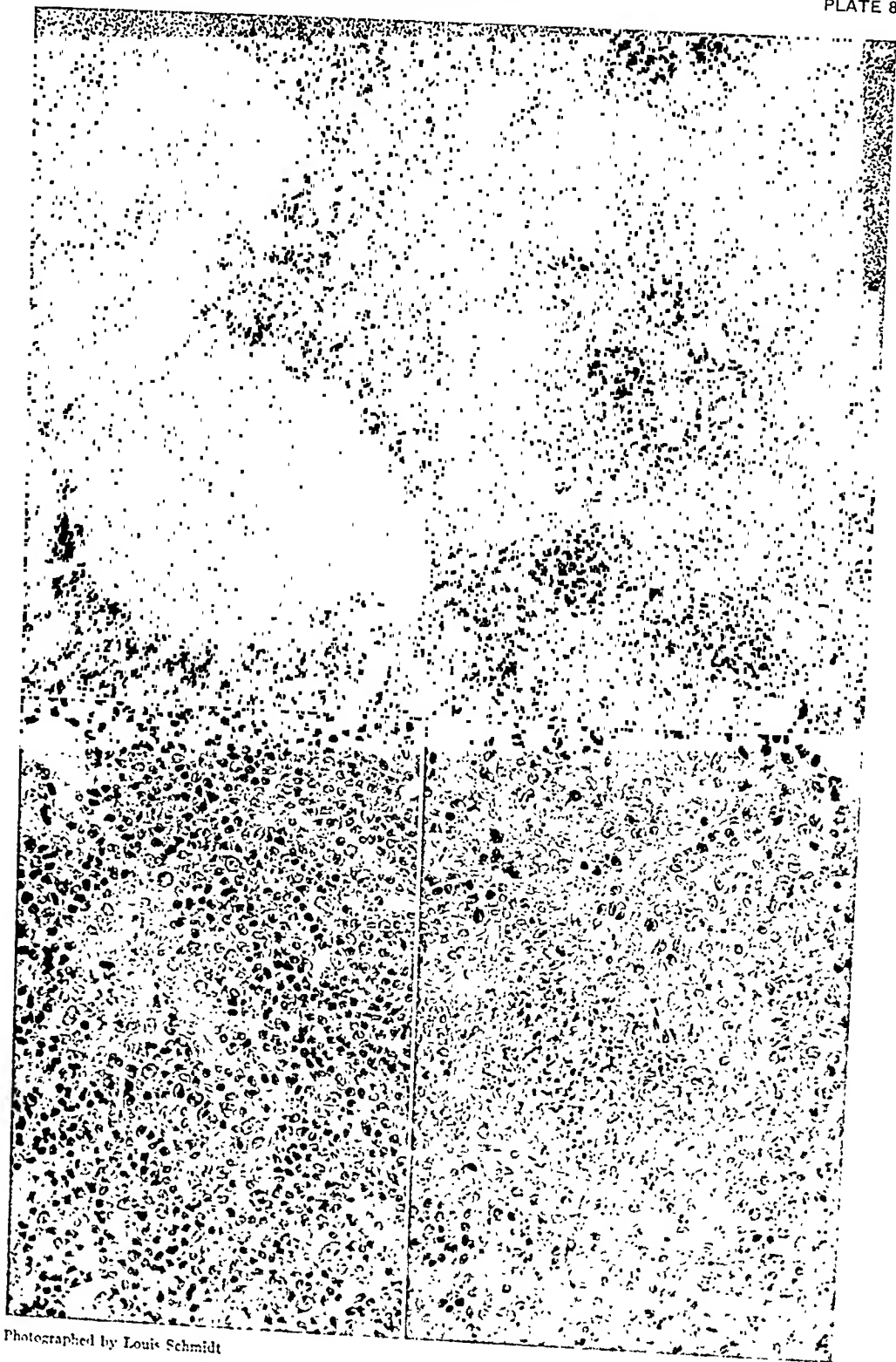
FIGS. 6 and 7. Early psittacosis lesions in liver; hyaline necrosis without much cellular infiltration. $\times 85$ and 375 . Eosin and methylene blue.

FIGS. 8 and 9. Lesions in liver caused by the virus of psittacosis; hyaline necrosis with infiltration of polymorphonuclear leucocytes. $\times 85$ and 375 . Eosin and methylene blue.

PLATE 10

FIGS. 10 and 11. Liver lesions late in the course of a psittacosis infection; areas of hyaline necrosis replaced by nests of mononuclear cells. $\times 100$ and 400 . Eosin and methylene blue.

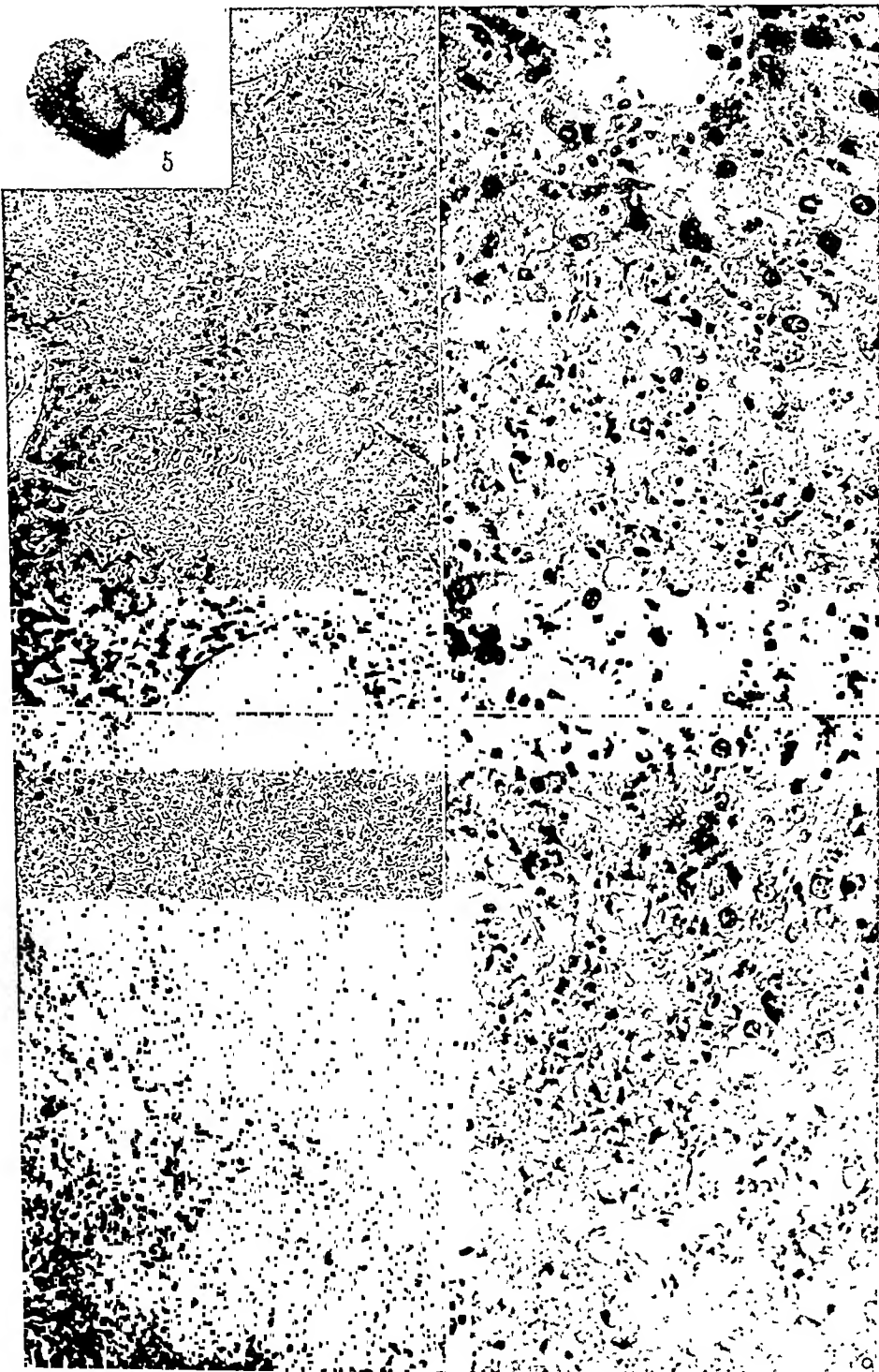
FIG. 12. Very extensive involvement of the liver that may appear early in the course of the infection; granular and fatty degeneration of the liver cells accompanied by nests of infiltrated cells. $\times 400$. Eosin and methylene blue.



Photographed by Louis Schmidt

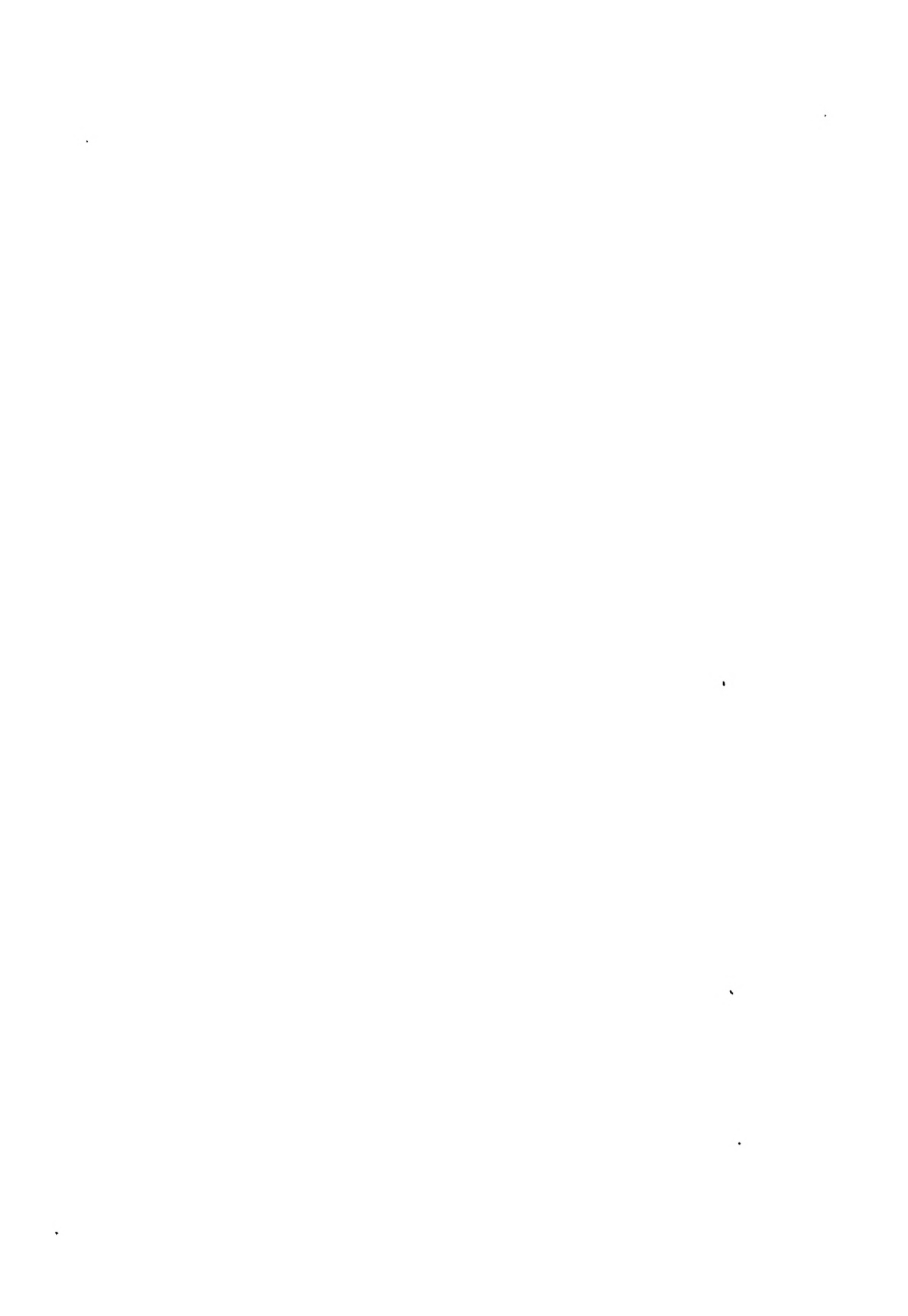


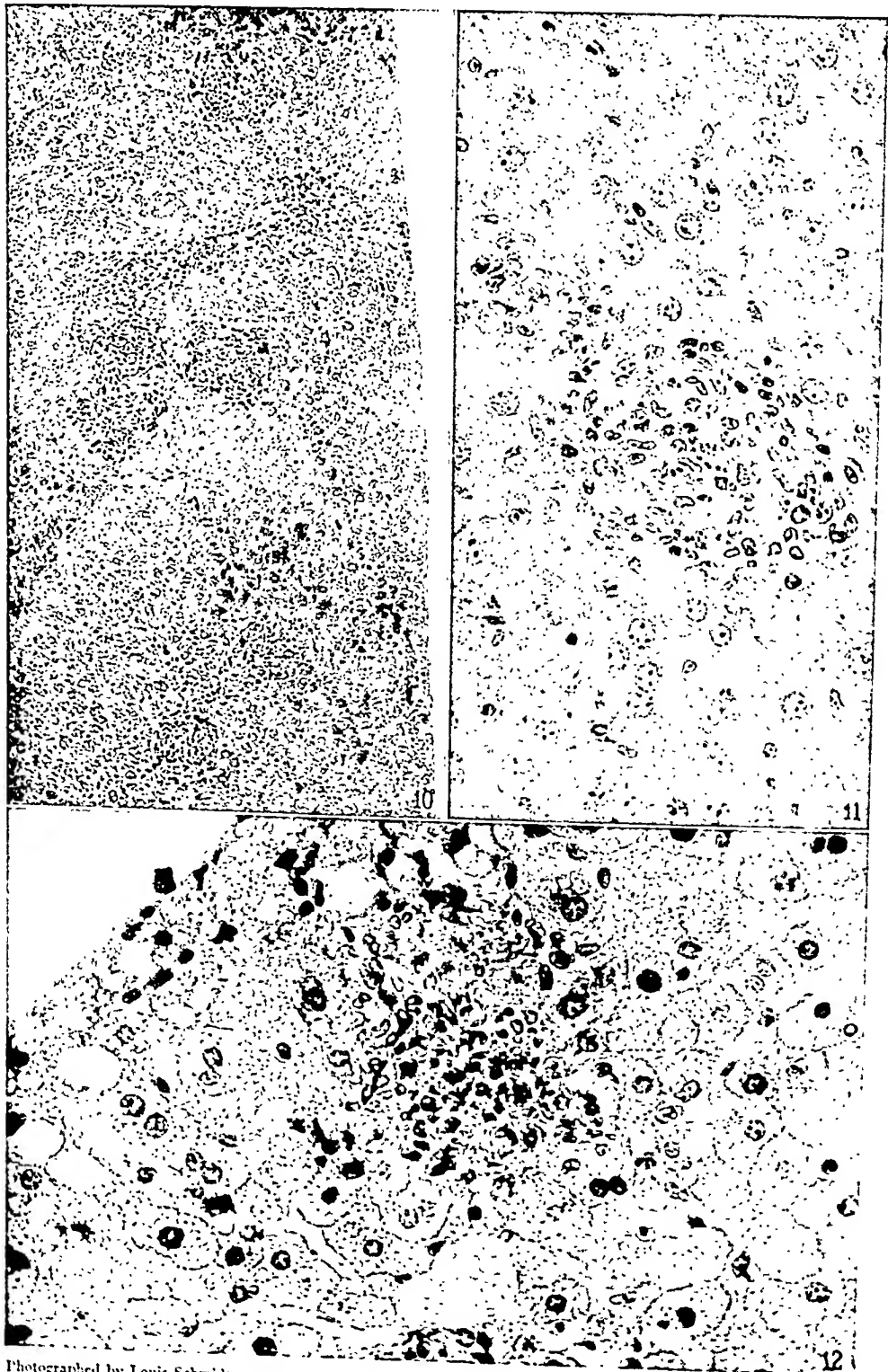
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Photographed by Louis Schmidt

(Rivers and Berry: Psittacosis. II)





Photographed by Louis Schmidt

PSITTACOSIS

III. EXPERIMENTALLY INDUCED INFECTIONS IN RABBITS AND GUINEA PIGS

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PLATE 11

(Received for publication, April 1, 1931)

When we began our investigations of psittacosis, the only animals known to be susceptible to the experimental disease were parrots, certain small birds closely related to parrots, and mice. Consequently it seemed desirable to ascertain whether other small laboratory animals are suitable for experiments with the disease. For this work, rabbits and guinea pigs were chosen. Results of the studies in which these animals were used and concerning which preliminary notes (1, 2) have already appeared are here set forth in detail.

Methods and Materials

Virus.—Virus from two sources was used; one strain came from an emulsion of the liver and spleen of Parrot N infected with virus originally obtained from a parrot with the natural disease, the other strain was derived from the liver and spleen of Parrot Wenz (WC) infected with material from a patient who had died of psittacosis. For more detailed information concerning these viruses, one may consult the first paper of this series.

Inoculations.—Intraperitoneal, intracutaneous, and corneal inoculations were used in a few instances. For the most part, however, the rabbits and guinea pigs were infected intracerebrally* and passages were accomplished by injections of 0.1–0.25 cc. of emulsified infectious brain tissue into the brains of normal rabbits and guinea pigs. The inoculations were made in the right half of the brain by means of a tuberculin syringe after the needle had been thrust through a small trephined opening in the skull. Transfers were made at the peak of the febrile reaction that usually came 3–7 days after inoculation.

* All operations were performed under ether anesthesia.

EXPERIMENTAL

Since symptoms and signs referable to the central nervous system constitute a conspicuous part of the clinical picture of psittacosis in man, and since many viruses are capable of propagation by means of brain to brain passages, it occurred to us that information concerning the etiological agent of psittacosis might be obtained by studying its effects on rabbits and guinea pigs infected intracerebrally. Furthermore, if it proved possible to establish the virus in these animals, an opportunity to investigate its distribution in their bodies would be provided. Moreover, we wished to determine whether passage of the virus through these more or less alien hosts would alter its pathogenicity for the natural host, the parrot, and for another alien host, the mouse. Finally, we desired to make certain investigations regarding immunity to psittacosis that would be facilitated in case the rabbit was found to be susceptible to the disease. Before discussing the results of our experiments, we shall give a general description of psittacosis experimentally induced by intracerebral inoculations of the virus in rabbits and guinea pigs. To this description, a few illustrative protocols are appended.

Clinical and Pathological Evidences of Infection

Following intracerebral inoculations of potent virus, all of the rabbits and guinea pigs, with a few exceptions, showed a high fever, *i.e.*, a temperature above 104°F. In many instances the temperature rose to 106–107°F, and occasionally reached 108.5°F. The peak of the febrile reaction was usually passed by the end of the 1st week after inoculation. During the febrile period, the animals appeared sick, refused to eat, lost weight extremely rapidly, and at times became ataxic. A few gravid pigs aborted. Some of the rabbits had convulsive seizures and died. As a rule, however, the animals recovered rapidly and appeared normal again 2 weeks after inoculation. The febrile reaction was so constant and striking that it was used as an index of infection.

The brains of rabbits and guinea pigs inoculated intracerebrally always showed pathological changes. The reaction was predominantly a meningitis characterized by infiltration of mononuclear and polymorphonuclear cells. The exudate was never extensive nor was it at any time purulent. The brain substance itself was not extensively damaged. Some degeneration of nerve cells and perivascular infiltration were seen. In the rabbits it was not always possible, because of the spontaneous encephalitis that occurs in them, to be certain of the cause of the changes around the vessels. In the pigs, however, there was no doubt that the virus of psittacosis produced perivascular infiltration.

The livers evidenced different degrees of enlargement and fatty degeneration. Some appeared to be almost normal, while others, particularly in the pigs, were so badly damaged that they had become chamois-colored and were very friable. In approximately 10 per cent of the animals, macroscopic evidences of necrosis or infarction (Figs. 1, 2) were found. Such necrotic or yellowish areas were occasionally surrounded by hemorrhagic zones. On section, no evidence of abscess formation was seen, and cultures of the tissues revealed no ordinary bacteria. Stained sections of the liver showed areas of necrosis somewhat similar to those seen in parrots and mice. There was no definite evidence of bile duct involvement. Occasionally vascular channels were thrombosed, but it was not possible to determine whether the vascular changes preceded or followed the necrosis. The splenic pulp was soft. The lungs, kidneys, and intestines were not particularly abnormal. Smears and sections of brains, livers, and spleens revealed none of the "minute bodies" found in parrots and mice infected with psittacosis.

Rabbit 2573, Mar. 28, 1930, was inoculated intracerebrally with 0.25 cc. of a brain emulsion from *Rabbit 2563*. Mar. 29, temp. 101.2°. Mar. 30, temp. 102.4°. Mar. 31, temp. 107°. Apr. 1, temp. 105.6°. Apr. 2, temp. 102.5°. Apr. 3, temp. 103.2°. Apr. 4, temp. 101.2°. The animal lost weight, but otherwise seemed in fair condition throughout the illness.

Rabbit 2610, Apr. 21, 1930, was inoculated intracerebrally with 0.25 cc. of a liver and spleen emulsion from *Rabbit 2606*. Apr. 22, temp. 104.6°, animal excitable. Apr. 23, temp. 103.8°. Apr. 24, temp. 107.5°, rabbit very irritable. Apr. 25, temp. 106.2°, animal in fair condition, killed with chloroform and autopsied immediately. Brain injected and slightly edematous. Lungs normal in appearance; liver enlarged and friable; spleen slightly enlarged with soft pulp; kidneys badly pitted as the result of an old nephritis; intestines injected. Aerobic and anaerobic cultures of the brain, lungs, liver, and spleen remained sterile.

Guinea Pig 2605, Apr. 14, 1930, was inoculated intracerebrally with 0.1 cc. of a brain emulsion from *Guinea Pig 2592*. Apr. 15, temp. 101.5°. Apr. 16, temp. 103.2°. Apr. 17, temp. 107.2°. Apr. 18, temp. 106°. Except for loss of weight and fever, the animal gave very little clinical evidence of infection. Sacrificed and autopsied immediately. Brain injected and edematous; lungs normal; liver large and yellowish as though it had undergone fatty degeneration; splenic pulp soft. Aerobic and anaerobic cultures of brain, liver, and spleen revealed no ordinary bacteria.

Guinea Pig 2607, Apr. 18, 1930, was inoculated intracerebrally with 0.1 cc. of a brain emulsion from *Guinea Pig 2605*. Apr. 19, temp. 103.2°. Apr. 20, temp. 106.4°. Apr. 21, temp. 107°. Apr. 22, temp. 105.8°. Loss of weight and fever were the only clinical evidences of infection noted. Animal sacrificed and autopsied immediately. Brain injected and edematous; lungs normal; liver had undergone fatty degeneration and over its surface were seen yellowish areas that appeared to be the result of focal necrosis or infarction; splenic pulp soft; kidneys normal. Aerobic and anaerobic cultures of brain, lungs, liver, and spleen remained sterile.

From the general description of the clinical findings and from the illustrative protocols it is obvious that the chief reactions in rabbits and guinea pigs to intracerebral inoculations of psittacosis virus are loss of weight and fever which at times may be excessively high. Most animals reveal little or no clinical evidence of involvement of the brain and quickly make a complete recovery. Apart from the mild meningo-encephalitis that all of the intracerebrally infected animals show, the chief pathological changes are noted in the liver where striking lesions are found in approximately 10 per cent of the cases. These alterations are characterized by fatty degeneration, focal necrosis, and infarct-like processes (Figs. 1, 2).

Results

Having detailed the clinical and pathological findings in rabbits and guinea pigs intracranially infected with psittacosis, we shall now describe the results of experiments on the serial passage of the virus in these animals.

Parrot N was infected by Krumwiede with a parrot strain of psittacosis virus and then transferred to The Rockefeller Institute. On Mar. 6, 1930, the bird died and at autopsy, a fatty liver, a large soft spleen, and a pericarditis were found. Cultures of the organs and pericardial exudate remained sterile, and on Mar. 8th 0.25 cc. of a 10 per cent liver and spleen emulsion were injected intracerebrally in a rabbit. The rabbit became sick and developed a high fever. On the 3rd day following inoculation, the animal was sacrificed and a 10 per cent brain emulsion was prepared and used to inoculate 2 normal rabbits intracranially. In this manner the virus was propagated serially through 5 groups of rabbits at which time, in addition to rabbits, a parrot and 4 mice were inoculated. All of these animals showed the clinical and pathological pictures of psittacosis. From a rabbit in the 10th set in the series, the virus was passed to normal rabbits by means of intracranial inoculations of emulsified liver and spleen. From a rabbit in the 12th set, blood was used to transfer the disease to a series of rabbits in which the virus was successfully propagated through 8 successive sets of animals.

From the 6th rabbit (Rabbit N₆) in the series of passages, 2 guinea pigs were inoculated (0.1 cc.) intracranially. From the brain of one of these pigs an emulsion was made and other pigs were infected intracerebrally in series. From a pig (Guinea Pig RN₆₅) in the 5th set of the series, 4 mice and a parrot were inoculated and developed typical psittacosis. From the liver and spleen of a pig in the 7th set, an emulsion was made and injected (0.1 cc.) into the brain of 2 pigs. These animals became sick and developed fever. From them the virus was passed to other pigs.

TABLE I

Summary of Reinoculation Experiments in Rabbits

Animal No.	1st inoculation Material Route, date	Virus in inoculum*	Strain of virus	Course after inoculation Highest T°	Time between inoculations days	Reinoculation Material Route, date	Strain of virus	Course after inoculation Highest T°	Autopsy	Active immunity + or -
McG	Human blood i.p. 3-7-30	-		Negative	40	Intracerebral inoculation with rabbit brain in manner identical with control Rabbit 2606 4-16-30	P	108 ^s Encephalitis, died	+	-
Old	Human blood i.p. 3-7-30	-		Negative	40		P	106 ^s Encephalitis, killed on 8th day	+	-
2534	Rabbit testicle, liver, and spleen i.c. and i.d. 3-12-30	+	P	106 ^s Tremulous and emaciated	35		P	Negative	Lived	+
Control 2606	Rabbit brain i.c. 4-16-30	+	P	108 ^s Wild, tremulous, killed on 5th day	77		P	Negative	Not done	+
	Autopsy showed typical psittacosis									
2562	Rabbit brain i.c. 3-24-30	+	H	104 ^s Emaciated	23	Rabbit brain i.c. 4-16-30	P	104 ^s Asymptomatic	Lived	±
2538	Parrot liver and spleen i.t. and i.d. 3-12-30	+	H	No fever, orchitis	65 78	Intracerebral inoculation with rabbit brain in manner identical with control Rabbit 2678 5-28-30	P P	Negative Negative	- -	++
2552	Guinea pig brain i.c. 3-18-30	+	P	105 ^s Tremulous	71		P	Negative	Not done	+

2574	Normal parrot liver and spleen i.c. and i.d. 3-29-30	-		Negative	60	Intracerebral inoculation with rabbit brain in manner identical with control Rabbit 2678 5-28-30			P	107 ^a Tremulous	+	-
2575	Normal parrot liver and spleen i.c. and i.d. 3-29-30	-		Negative	60				P	107 ^a Wild and tremulous	+	-
2577	Parrot liver and spleen i.c. and i.d. 3-29-30	+	P	104 ^s Asymptomatic	60				P	106 ^s Tremulous diarrhea	+	-
2579	Rabbit brain i.c. 3-31-30	+	H	Negative	58				P	107 Tremulous, diarrhea Negative	Not done	-
2603	Rabbit brain i.c. 3-31-30	+	H	106 ^s Asymptomatic	44				P	Negative	-	+
2622	Rabbit brain i.c. 4-14-30	+	P	106 ^s Emaciated	34				P	Negative	-	+
2624	Rabbit brain i.c. 4-24-30	+	P	104 ^s Tremulous	33				P	Negative	-	+
2628	Rabbit brain i.c. 4-25-30	+	H	106 Encephalitis	32				P	Negative	-	+
Control 2678	Rabbit brain i.c. 5-28-30	+	P	106 ^s Tremulous, killed on 5th day		Autopsy showed typical psittacosis						

* Presence or absence of virus in inoculum determined in addition by other animal inoculations.

(intramuscularly and intraorally) to a parrot that sickened and died of psittacosis. From this bird, 2 other parrots were successfully infected by means of nasal secretions and feces respectively.

The work described above, some of which is schematically portrayed in Text-fig. 1, indicates that the virus of psittacosis is capable of indefinite propagation by means of brain to brain inoculations in rabbits and guinea pigs. Furthermore, it is evident that 5 such passages of the virus through rabbits, followed by 5 additional serial passages in guinea pigs, did not cause the active agent to lose its pathogenicity for parrots and mice. Moreover, the presence of the virus in rabbit's blood, liver, and spleen, and in guinea pig's liver and spleen was demonstrated. Finally, 2 strains of virus, one from a parrot and the other from man, affected rabbits and guinea pigs in a similar manner.

Immunity

Parrots upon recovery from an attack of psittacosis possess an active immunity (see first paper of this series), while mice may not (see second paper). It seemed important, therefore, to ascertain whether rabbits react to a primary infection with the virus by the development of an active immunity, and, if they do, to study by immunological methods the relation of the 2 strains of active agent (parrot and human) under investigation.

Fourteen rabbits that had received different materials intraperitoneally, intracerebrally, intradermally, and intratesticularly were reinoculated intracerebrally at varying periods of time, 23-78 days, following the first injections. Some of the primary inocula contained no virus, others had the parrot strain, while yet others carried the human strain. In each instance, the parrot strain was used for reinoculation. Of the 6 rabbits (Table I) that received no virus at the time of the first inoculation, all developed high fever and were sick following reinoculation with the virus. Of the 8 rabbits, however, that received virus in the primary inoculum, only 1 showed fever. This animal was first reinoculated 23 days after its primary infection. In contrast, 65 days after receiving its original dose of virus, it was reinoculated a second time and was found to be fully resistant.

The results of the reinoculation experiments summarized in Table I clearly show that rabbits upon recovery from psittacosis are actively immune. Furthermore, it is evident that the 2 strains of virus under investigation are immunologically similar.

Since the rabbit responds to intracerebral inoculations of the virus with a striking rise of temperature, it occurred to us that its febrile reaction might be used as an index of infection, thus indicating the state of activity of virus in neutralization tests. Examination in which the virus serum mixtures were tested in the brains of rabbits are described below.

Serum was collected from a patient on the 2nd day of the disease and also on the 10th day after subsidence of fever. A normal individual who had never had psittacosis was bled for control serum. Brain emulsion containing virus was diluted 1:5, 1:10, 1:20. Portions of these dilutions were mixed with equal amounts respectively of the 3 specimens of serum. The mixtures were allowed to stand 1 hour at 37°C. and then 0.25 cc. of each were injected intracerebrally in a rabbit. Thus 9 rabbits were inoculated. In one instance only, neutralization of the virus by convalescent serum appeared to have occurred. When the experiment was repeated, however, no evidence of inactivation of the virus by convalescent serum was obtained.

From the results of the neutralization experiments described above, one is not justified in concluding that convalescent human serum shows any appreciable amount of neutralizing power. These findings are similar to those obtained when mice (see second paper of this series) are employed as the test animal.

DISCUSSION

We have reported the results of our work dealing with the propagation of the virus of psittacosis in the brains of rabbits and guinea pigs. Other workers (3, 4) have confirmed our observations, and, in addition, have been able to obtain similar results by means of intratesticular inoculations. Furthermore, they have shown that the skin of rabbits and guinea pigs is susceptible to the virus and can be used for titrations of its activity. Moreover, the failure of human convalescent serum to inactivate virus has been observed by other workers (4, 5) as well as by ourselves. Thus, no experimental proof has been adduced to show that convalescent serum is of value in the treatment of psittacosis.

CONCLUSIONS

1. Rabbits and guinea pigs are susceptible to psittacosis virus introduced intracerebrally. By means of brain to brain passages in these animals the active agent is capable of propagation indefinitely.

2. Serial passages of the virus through rabbits and guinea pigs do not cause the active agent to lose its pathogenicity for parrots and mice.

3. The chief clinical evidences of infection in rabbits and guinea pigs following intracranial inoculation of the virus are fever and loss of weight. The pathological changes are characterized by a mild meningo-encephalitis, and fatty degeneration, focal necrosis, and infarction of the liver.

4. Rabbits upon recovery from an attack of psittacosis are actively immune.

5. Two strains of virus, human and parrot, were found to be immunologically similar.

6. No evidence was obtained to show that human convalescent serum possesses an appreciable amount of neutralizing substances.

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EXPLANATION OF PLATE 11

FIG. 1. Liver of a guinea pig, infected with psittacosis, showing fatty degeneration and areas of necrosis or infarction. $\times 1$.

FIG. 2. Liver of a rabbit, infected with psittacosis, showing areas of necrosis or infarction. $\times 1$.



1



2

PSITTACOSIS

IV. EXPERIMENTALLY INDUCED INFECTIONS IN MONKEYS

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PLATES 12 TO 17

(Received for publication, April 1, 1931)

In the three papers immediately preceding this one, psittacosis experimentally induced in parrots, rabbits, guinea pigs, and mice was described. None of the animals employed in the work detailed, however, evidenced pulmonary signs or lesions of any significance. Inasmuch as psittacosis in man manifests itself chiefly by pathological changes in the lungs, and since no one had employed monkeys for the experimental study of the malady, we decided to determine whether it is possible to produce in certain lower primates pulmonary lesions similar to those found in human beings infected with the virus of psittacosis. In this study, 6 experiments were performed in which 12 monkeys were inoculated one or more times.

Methods and Materials

Virus.—The psittacosis virus was obtained from the livers and spleens of Wenz mice (see second paper of this series) carrying a human strain of the active agent. The emulsions containing the virus for each experiment were shown to be free from ordinary aerobic and anaerobic bacteria.

Animals.—Healthy medium sized Indian monkeys (*Macacus rhesus*) proved to be satisfactory for the work. Only animals whose lungs were shown by X-ray examination to be normal were used.

*Inoculations.**—The majority of monkeys was infected by intratracheal inoculations (1–4 cc.) accomplished by thrusting a small needle into the trachea below the larynx and then injecting the emulsion with a syringe. 2 monkeys were inoculated intracerebrally (1 cc.) through a small trephined opening in the skull. 2 monkeys received the infectious agent by intranasal instillations (1–2 cc.) of organ

—
All operations were performed under ether anesthesia.

EXPERIMENTAL

The objects of the first experiment were to determine whether monkeys are susceptible to the virus of psittacosis injected intratracheally or intracerebrally, and to ascertain whether the virus can be propagated by serial passages in these animals.

Experiment I

Monkey A, May 20: Temp. before inoculation not taken. Received intratracheally 2.5 cc. of liver and spleen emulsion from mice WC₁₇. *May 22*, temp. 103.8°, appears normal. *May 23*, temp. 105.4°, condition same. *May 24*, temp. 104.8°. *May 25*, temp. 104°. *May 26*, temp. 105°, seems sick and eating poorly. X-ray of chest shows a shadow extending to left of heart and mottling of both lower lobes behind the diaphragm. *May 27*, temp. 103.4°, still appears sick. *May 28*, temp. 102.6°, condition improved. X-ray of chest reveals an extension of the shadows noted on the 26th. Animal killed with chloroform. *Autopsy:* Heart, pericardium, liver, spleen, and kidneys appear normal. Lungs: No pleurisy. Both lower lobes are partially consolidated and have taken on a lilac-pink color. The other lobes on the right are involved in a patchy manner. The consolidated lobes on section appear homogeneous, smooth, free of edema. The bronchi are not raised above the cut surface and contain no exudate. The hilar lymph glands are enlarged and contain a few small hemorrhages. Smears from the lungs showed no ordinary bacteria, and none of the "minute bodies" found in parrots and mice infected with psittacosis virus. Cultures, aerobic and anaerobic, of the lungs and liver remained sterile. Pieces of involved lung were emulsified and injected into 4 mice intraperitoneally and into Monkey E intratracheally. 2 of the mice died 6 days after inoculation and showed typical psittacosis lesions in the liver and spleen. Numerous "minute bodies" were found in smears from these mouse organs. The other 2 mice were sick for a number of days and finally died of psittacosis.

Monkey E, May 29: Temp. before inoculation, 101.8°. X-ray of chest negative. Received intratracheally 4 cc. of lung emulsion from Monkey A. *May 30*, temp. 102°, animal well. *May 31*, temp. 104°, seems sick. *June 1*, temp. 105°, sick. *June 2*, temp. 103.6°, sick and has diarrhea. X-ray of chest shows no obvious lesions. *June 3*, temp. 103°. *June 4*, temp. 105.8°, diarrhea persists. X-ray of chest negative. *June 5*, temp. 104.4°. *June 6*, temp. 102.5°, animal still has diarrhea. X-ray of chest negative. Killed with chloroform and autopsied immediately. Pericardium contains 1 cc. of sticky greyish exudate; no "minute bodies" or bacteria found in smears; cultures sterile. Right lung bound down by fresh fibrinous adhesions. 3 upper lobes normal. Right lower lobe shows scattered subpleural hemorrhages. On section several small greyish nodules noted in the parenchyma. Hilar lymph nodes enlarged and hemorrhagic. Liver has a small yellowish area near the point where the round ligament emerges. Cultures from

the lungs and liver sterile; no "minute bodies" found in smears from these organs. An emulsion from the liver was injected into 4 mice, 2 of which died 9 days later. The other 2 died 17 and 30 days respectively after inoculation. All the mice had psittacosis.

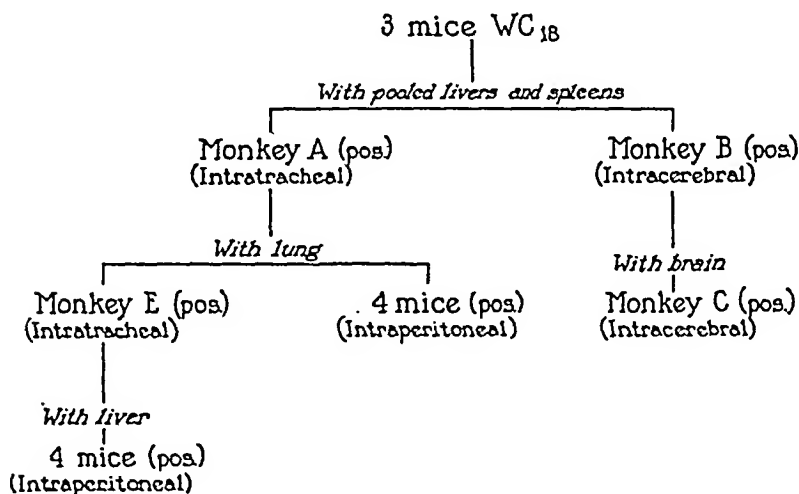
Monkey B, May 20: Received in the brain 0.75 cc. of an emulsion similar to that given Monkey A. *May 22*, temp. 104°, animal sick, weak, and has diarrhea. Was observed during a convulsion. *May 23*, temp. 104.5°, weak, ataxic; severe diarrhea. *May 24*, temp. 105°, condition worse. *May 25*, temp. 103.8°. Animal weaker and more ataxic. *May 26*, temp. 99°. Unconscious and having repeated convulsive seizures. Killed with chloroform and autopsied immediately. Lungs, liver, and spleen are negative. Brain injected and edematous. Cultures from brain and other organs sterile. Smears negative for the "minute bodies." Stained sections of the brain showed a mild encephalitis with some degeneration of nerve cells. The predominant lesion was a meningeal reaction characterized by mononuclear infiltration.

Monkey C, May 28: Received intracerebrally 0.75 cc. of a brain emulsion from Monkey B. *May 29*, temp. 102.8°. Animal well. *May 30*, temp. 102.6°. *May 31*, temp. 103°. *June 1*, temp. 102.6°. Animal still seems well. *June 2*, temp. 105°. *June 3-7*, temp. 104° or above. Animal definitely sick. X-ray of chest taken on June 4 negative. *June 8-14*, temp. 102-102.6°. Monkey appears normal again.

The results of Experiment I detailed in the protocols above and portrayed in Text-fig. 1 indicate that macaques are susceptible to the virus of psittacosis and that the virus can be passed from monkey to monkey by intratracheal or by intracerebral inoculations. There are several points of interest, however, that should be noted. The reactions in the first monkeys of the 2 series were much more severe than were those in the second lot of animals. Moreover, mice inoculated with emulsions of Monkey A's lung and Monkey E's liver developed psittacosis, but they died more slowly than did mice inoculated with mouse passage virus. These facts suggest the possibility that passage of the virus through monkeys alters it in such a manner that transfers from monkey to monkey become relatively difficult. Similar conditions may hold for human beings who seem to be quite susceptible to the virus emanating from parrots, but relatively non-susceptible when exposed to the disease in man. Attention should also be directed to the fact that monkeys receiving the virus intracerebrally developed no pulmonary lesions. Thus, it appears that the portal of entry of the virus profoundly influences its localization and the pathological changes

caused by it in these animals. This fact lends evidence in favor of the idea that the active agent enters man, in whom involvement of the lungs usually occurs, through the upper respiratory tract. Finally, the "minute bodies" found in parrots and mice infected with psittacosis virus were not seen in the lungs and brains of monkeys attacked by the same active agent. Yet emulsions of these organs produced psittacosis in mice, and smears from their livers and spleens showed the small bodies. Failure to find these structures in monkeys, however, does not necessarily mean that none were present.

Monkey experiment 1



TEXT-FIG. 1. Diagrammatic representation of Experiment I

Before proceeding it seemed advisable to repeat part of the first experiment for confirmatory purposes.

Experiment II

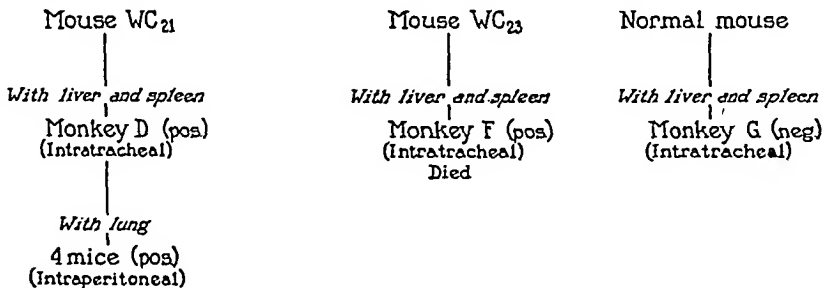
Monkey D, May 28: Temp. 102.4°. X-ray of chest negative (Fig. 11). *May 29,* animal received intratracheally 4 cc. of pooled liver and spleen emulsion from mice WC₂₁. *May 30,* temp. 103.8°, seems well. *May 31,* temp. 105°, eating poorly. *June 1,* temp. 104.5°, sick. *June 2,* temp. 104.8°, slight cough. X-ray of chest reveals involvement of a large part of the right lung and of the left lower lobe (Fig. 12). Killed with chloroform and autopsied immediately. No evidence of pleurisy. The 4 lobes of the right lung and the two lower lobes of the left reveal a hemorrhagic, purplish consolidation spreading out from the hilum. On section, the surface is smooth and dry, similar to the cut surface of meat. No exudate in

bronchi. Free, straw-colored fluid in pericardium. Liver, spleen, intestines, adrenals, and kidneys appear normal. In smears from the lungs and pericardial fluid no "minute bodies" were found. Cultures of lungs and liver sterile. 4 mice were inoculated (0.5 cc. each) intraperitoneally with a lung emulsion from Monkey D. All died of psittacosis; 1 on the 3rd, 1 on the 6th, 1 on the 15th, and 1 on the 18th day respectively after inoculation.

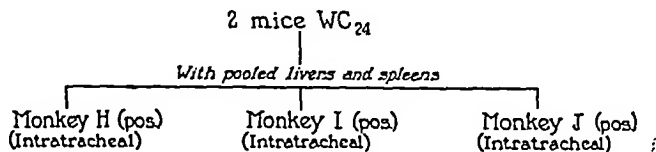
The results of Experiment II, shown in Text-fig. 2, confirm those obtained in the first experiment. Having determined that liver and spleen emulsions from mice infected with psittacosis cause a consolidation

Monkey experiment 2

Monkey experiment 3



Monkey experiment 4



TEXT-FIG. 2. Diagrammatic representation of Experiments II, III, and IV

tion of the lungs, we decided to see what effect similar emulsions from normal mice would have. This was undertaken in Experiment III.

Experiment III

Monkey F received intratracheally 2.5 cc. of an emulsion of pooled livers and spleens from psittacosis mice WC₂₃, while Monkey G received in a similar manner 2.5 cc. of an emulsion of pooled livers and spleens from normal mice.

Monkey F, June 2: Temp. 101.6°. X-ray of chest negative. *June 3,* date of inoculation. *June 4,* temp. 106.2°, seems well. *June 5,* temp. 105.2°, stools loose. *June 6,* temp. 104.6°, sick, has cough. X-ray of chest shows extensive shadows in both lower lobes behind the dome of the diaphragm and also in the

right middle and upper lobes. *June 7*, temp. 104°, considerable difficulty in breathing. X-ray indicates that the middle lobe on the left side is also involved. *June 8*, temp. 104.8°, very sick, does not eat, has difficulty in breathing, stools loose. *June 9*, temp. 102.2°. X-ray shows large shadows throughout the right side with extension of the pneumonia in left lower and middle lobes; the left upper only remains clear. Shortly after the X-ray was taken the monkey died and was autopsied immediately. Lungs: No pleurisy; many subpleural hemorrhages. Right: The upper lobe with the exception of the edges is completely consolidated. The two middle lobes are less completely involved, the edges being quite free. The lower lobe is almost completely consolidated. Left: Upper and middle lobes are only slightly involved. Lower lobe is almost completely consolidated, edges alone remaining free. On section the consolidated lobes do not have the appearance usually seen in ordinary lobar pneumonia. They are not granular, have a meaty appearance with slight peribronchial areas of pallor. No exudate is noted in the bronchi. Heart: 3 or 4 cc. of clear straw-colored fluid in pericardium. Liver: Enlarged, edges rounded. Near where the round ligament emerges there appears to be a small infarct. The liver has a peculiar appearance suggesting widespread fatty degeneration with areas of necrosis. Spleen: Pulp very soft. Adrenals and kidneys appear normal. Cultures of the lungs, liver, and pericardial fluid sterile. Smears for the "minute bodies" negative.

Monkey G was treated in a manner similar to that in which F was handled, with the exception that an emulsion of normal spleens and livers was used as an inoculum. The animal evidenced no signs of illness, never had any fever, and 4 X-rays of the chest revealed no areas of consolidation.

The results of the above experiment (Text-fig. 2) indicate that an emulsion of livers and spleens from normal mice introduced intratracheally does not produce a pneumonia in monkeys. In the next experiment, No. IV, 3 monkeys received intratracheally psittacosis virus in an emulsion (2 cc. to each animal) of pooled livers and spleens from mice WC₂₄. The animals, having been watched and examined frequently by means of the X-ray were killed 2, 6, and 13 days respectively after inoculation. In this way lungs were obtained during different stages of the disease in order to study the evolution of the pathological process.

Experiment IV

Monkey H, June 3: Temp. 103.5°. X-ray of chest negative. *June 5*, temp. 103.2°. *June 6*, temp. 102.4°. *June 7*, date of intratracheal inoculation. *June 8*, temp. 105.3°, diarrhea. *June 9*, temp. 104.8°, diarrhea persists. X-ray of chest shows extensive mottling of right lower lobe with smaller shadows in the left lower. There is evidence of spreading of the infection along the bronchial tree into the

upper and middle lobes (Fig. 1). Animal killed with chloroform and autopsied immediately. Pericardium, heart, spleen, kidneys, and adrenals seem to be normal. A small whitish area in the edge of one lobe of liver near the round ligament. Lungs show lesions spreading out from the hilum along the bronchial tree into all the lobes. The involved portions are purplish pink, only partially consolidated, but sharply demarcated from the surrounding normal lung tissue. Cultures of the lungs sterile.

Monkey I, June 3: Temp. 102.5°. X-ray of chest negative (Fig. 5). *June 5,* temp. 103°. *June 6,* temp. 102.4°. *June 7,* date of intratracheal inoculation. *June 8,* temp. 105°, eating poorly. *June 9,* temp. 103.5°, sick and weak. X-ray shows small shadows in both lower lobes near the hilum (Fig. 6). *June 10,* temp. 103.2°, condition unchanged. X-ray: Shadows in both lower lobes have extended toward the periphery. The 2 middle lobes and possibly the upper on the right side are consolidated near the hilum. *June 11,* temp. 104.4°, condition worse. X-ray: The areas of consolidation have increased in size and the shadows are more intense. *June 12,* temp. 104.8°, condition worse. X-ray: Shadows indicate that the areas of consolidation have almost reached the periphery (Fig. 7). *June 13,* temp. 103.5°. X-ray shows no change. *June 14,* temp. 105°, still quite sick and has lost weight. *June 15,* temp. 104.2°. *June 16,* temp. 103.4°, better and eating a small amount of food. X-ray: Some clearing of the shadows at the periphery of the lesions (Fig. 8). *June 18,* temp. 100.8°, much better. X-ray: Marked clearing of the shadows (Fig. 9). *June 20,* temp. 102.4°, animal seems almost normal again. X-ray: A few irregular shadows persist near the hilum (Fig. 10). Monkey killed and autopsied immediately. Heart and pericardium normal. Both pleural cavities clear. Lungs: The upper and 2 middle lobes on the right bound to each other by loose adhesions. Only slight changes near the hilum are noted in the upper and one of the middle lobes, while in the other middle and lower lobes quite firm areas are still detected near the hilum around the vessels and bronchi. On the left side, the upper lobe is normal, while near the hilum areas of consolidation in the middle and lower lobes still exist. These are less extensive than are those in the right lower. On section the involved lobes exhibit air-containing alveoli around the periphery, while in the central portions along the bronchi and vessels a yellowish gray semiconsolidated tissue, dry and relatively smooth appearing, is found. Hilar lymph glands not greatly enlarged. Liver shows fatty degeneration. Kidneys and spleen normal in appearance. Hemorrhage in medulla of right adrenal gland. Cultures of lungs: One remained sterile, the other showed a few indifferent streptococci.

Monkey J, June 5: Temp. 104.3°. X-ray of chest negative. *June 7,* date of intratracheal inoculation. *June 8,* temp. 105°, seems well. *June 9,* temp. 104.5°, condition unchanged. X-ray: Shadows in both lower lobes near the hilum. *June 10,* temp. 104.2°, sick, weak, not eating. X-ray: All lobes on the right seem to be involved near the hilum; extension of shadow in left lower lobe. *June 11,* temp. 105.6°, worse. X-ray: Increase of shadows on both sides, but left upper and

middle lobes still relatively clear. *June 12*, temp. 104.6°, condition same. X-ray: No change in shadows. *June 13*, temp. 103.2°, animal still very sick and has difficulty in breathing. X-ray: The right lung is almost completely consolidated, the left upper and middle lobes remain relatively clear (Fig. 4). Animal killed with chloroform and autopsied immediately. The lobes on the right side, bound to each other and to the chest wall by a sticky mucoid fibrinous exudate, are completely consolidated with the exception of the peripheral portions. Slight amount of pleurisy on the left side with extensive consolidation of the lower lobe; the other 2 lobes relatively free. On section the color and consistency of the lungs similar to that described in other monkeys. Hilar lymph glands enlarged. Heart enlarged. In the pericardial sac are 1 or 2 cc. of sticky, whitish exudate. Liver shows a small area of necrosis near the round ligament. The intestines, spleen, kidneys, adrenal glands, and brain appear normal. In the different exudate and organs, no bacteria or "minute bodies" were found, and cultures from them remained sterile.

In Experiment IV, by means of X-ray examinations that were confirmed by autopsy findings, it was possible to follow the spread of the consolidation from the hilum towards the periphery and to see it regress in the reverse order. The course of events, shown by this set of photographs to occur in monkeys infected with psittacosis virus, parallels that observed in the lungs of man infected with the same active agent (1). Having demonstrated conclusively that a pneumonia in monkeys regularly follows the intratracheal inoculation of psittacosis virus, we considered it essential to ascertain, Experiment V, whether a pulmonary infection occurs when the inciting agent is instilled intranasally.

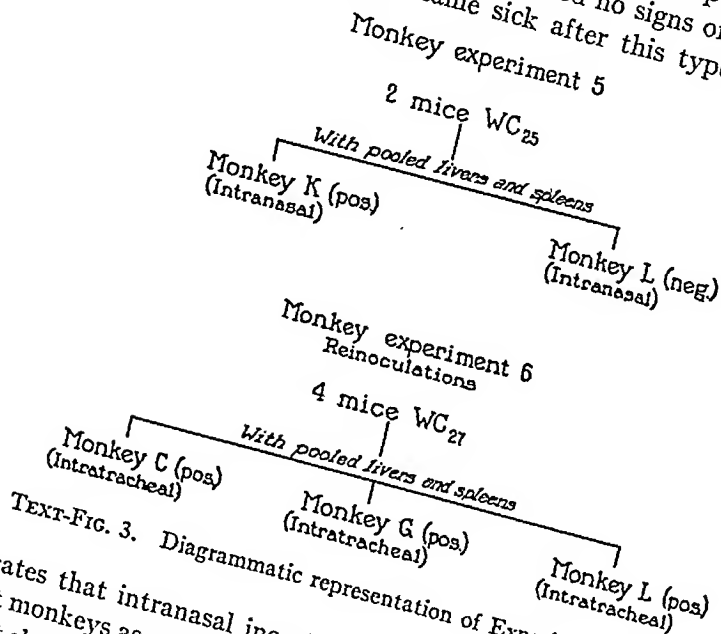
Experiment V

Into the noses of Monkeys K and L respectively, were instilled 1.5 cc. of an emulsion of pooled livers and spleens from psittacosis mice WC₂₅.

Monkey K, June 10: Temp. 101.6°. X-ray of chest negative (Fig. 2). *June 11*, date of intranasal inoculation. *June 12*, temp. 102.8°. *June 13*, temp. 105.4°, animal well. X-ray: Chest still clear. *June 14*, temp. 105°. X-ray: Beginning shadow at hilum on the right side, and slight mottling of right lower lobe. *June 15*, temp. 105.6°, eating poorly, slight diarrhea. *June 16*, temp. 105°, less diarrhea. X-ray: Shadow at hilum has increased and is spreading in a manner similar to that observed in man (Fig. 3). *June 17*, temp. 104.2°, animal does not appear very sick. X-ray shadows more intense. *June 18*, temp. 104.6°, condition same. X-ray shadows unchanged. Animal killed with chloroform and autopsied immediately. All organs appear normal with the exception of the right lung. In the lower portion of the upper lobe and at the top of the lower lobe are areas of

consolidation surrounding the main bronchi. On section, the surface of the consolidated tissue is dry, gray, and smooth. Cultures sterile.
 Monkey L received an inoculum similar to that of Monkey K, but at no time did it have fever, or pulmonary consolidation as evidenced by repeated X-ray photographs.

Of the 2 monkeys receiving intranasal inoculations of psittacosis virus as described in the above experiment, 1 developed a typical pneumonia with fever while the other showed no signs of infection. The fact that only 1 animal became sick after this type of inoculation



TEXT-FIG. 3. Diagrammatic representation of Experiments V and VI indicates that intranasal inoculations with psittacosis virus will not infect monkeys as regularly as do intratracheal injections. Nevertheless, it shows that, when the virus does enter in such a manner a pneumonia similar to that seen in man occurs. Thus, experimental evidence has been adduced in favor of the idea that man usually becomes infected through the upper respiratory tract. The last experiment (No. VI, see Text-fig. 3), in which monkeys were used, consisted of reinoculations of Monkeys C, G, and L. Monkey C had recovered from an intracerebral inoculation administered 26 days previously; Monkey G had been given an intratracheal injection

of an emulsion of livers and spleens from normal mice; Monkey L had had virus instilled in the nose 12 days previously without any evidence of infection. Each animal was reinoculated intratracheally with 1 cc. of mouse virus, and all responded with a moderately severe pneumonia that was verified by X-ray examinations and autopsies. Monkey C was the only animal in the group that had previously evidenced signs of infection due to psittacosis virus. Consequently, it was the only one that might have been expected to show an immunity to reinfection. It did not, however. Whether this lack of immunity was due to the type of the first infection (intracerebral), whether 26 days between the first and second inoculations was too short a period of time for a demonstrable immunity to appear, or whether monkeys do not develop a solid immunity against psittacosis virus are questions that cannot be answered at present.

Microscopic Pathology

In the three preceding papers the pathological changes induced by psittacosis virus in parrots, mice, rabbits, and guinea pigs were described. Significant lesions, however, were not found in the lungs of these animals. Moreover, pulmonary lesions experimentally induced by the action of the virus in such hosts have been described by only a few workers, and in each instance the picture presented leaves one in doubt as to whether or not the morbid changes were caused by the active agent under discussion. In the present communication, the clinical and gross pathological pictures of psittacosis pneumonia in monkeys have been recorded. It now remains to describe the microscopic pathology of the pulmonary changes. Inasmuch as the animals were sacrificed after different intervals of time (2-13 days) had elapsed following inoculation, an excellent opportunity for a study of all stages of the morbid conditions in the lungs was presented. Therefore, the monkeys will be discussed in the order in which they were killed. There is, however, at least one fallacy in this mode of presentation, *viz.*, after an infection in the lungs has been under way for several days, early and advanced lesions may be found in the same animal. This fact must be borne in mind.

Two Days after Inoculation.—Monkey H. Examination of sections of whole lobes reveals that the consolidation first appears around the vessels and bronchi

near the hilum. Extending from the small areas of consolidation the walls of the alveoli (Fig. 15) are thickened and edematous, and show evidence of cellular infiltration. Study of many such sections leads one to suspect that the infection spreads along the alveolar walls. In many areas these structures are engorged with blood and contain a few polymorphonuclear cells (Fig. 16). In other places they are thickened by the swelling of alveolar cells and the infiltration of mononuclear elements (Fig. 17). Serous exudate (Fig. 16), fibrin, and desquamated cells are seen in many alveoli, while in definitely consolidated areas some of the alveolar spaces are filled with cells predominantly of the polymorphonuclear type (Fig. 18).

Four Days after Inoculation.—Monkey D. Sections of entire lobes again show that the consolidation begins near the hilum and spreads peripherally. In Fig. 20, the alveoli near the pleura are distended with serous exudate in which are embedded a few cells. In more centrally located areas, the number of cells in the exudate increases, and the alveolar walls are thickened (Fig. 21). As the hilum is approached, completely consolidated lung is encountered, in which some of the alveolar walls appear necrotic and the alveolar spaces are filled with a mixture of polymorphonuclear and large mononuclear cells (Fig. 22), while other areas reveal thickened alveolar walls and alveoli distended entirely by mononuclear cells (Fig. 24). Small hemorrhages and masses of fibrin are scattered throughout the involved portions of the lung. The origin of the mononuclear cells—there seems to be more than one kind present (Fig. 23)—in the exudate is not definitely known.

Six Days after Inoculation.—Monkeys F and J. The pictures presented by these monkeys are similar to those found in sections of Monkey D. Some differences, however, may be noted, for example, the infrequency of polymorphonuclear cells, and the appearance in the alveolar walls in certain places of giant cells and mitotic figures.

Seven Days after Inoculation.—Monkeys K and L. At this stage of the infection, practically no polymorphonuclear cells are found, hemorrhages are rarely seen, fibrin and serous exudates are still present in places, and a distinct perivascular cuffing with mononuclear cells, many of which are distended with pigmented granules, has made its appearance. This phenomenon of cuffing is noted in earlier monkeys but not to such an extent as in F and J and the others to follow. Evidences of cellular proliferation in the alveolar walls are pronounced and in many places it is difficult to distinguish these structures from the exudate or cells in the alveolar spaces (Fig. 19—Monkey K inoculated intranasally).

Eight Days after Inoculation.—Monkey A. This animal was killed on the 8th day after inoculation, the 1st day that an improvement in its condition was noted. An attempt to describe the microscopic pathology observed in the lungs will not be made, since no description can equal the pictures presented in Figs. 25, 26, and 27, an examination of which will show the condition of the alveolar walls and the contents of the alveolar spaces, and justify the conclusion that the pathological changes present are not those usually encountered in lobar pneumonia of man and monkeys.

Ten and Twelve Days after Inoculation.—*Monkeys C and G.* Clinically these animals were recovering from their pneumonia. Examination of the sections reveal evidences of resolution that will be discussed in connection with the next animal (Monkey I).

Thirteen Days after Inoculation.—*Monkey I.* This animal was killed 13 days after inoculation, 4 days after it began to show clinical evidences of improvement, and at a time when the X-ray photographs had almost returned to normal (Figs. 5–10). Grossly the lungs reveal more evidences of consolidation than had been expected from the X-ray pictures. Examinations of stained sections of whole lobes confirm the macroscopic pathology. Areas of dense tissue (Fig. 28) in which it is difficult to distinguish alveolar walls from alveolar contents are seen. Such areas are separated from each other by large air-containing spaces or distended alveoli (Fig. 29). A superficial examination of sections stained with eosin methylene blue suggests that a great deal of organization is taking place; but by the use of Mallory's aniline blue and orange G stain (Figs. 29 and 30) it becomes evident that only a slight amount of connective tissue is being laid down and that the alveoli are filled with large mononuclear cells. It is also obvious that resolution is occurring from the periphery towards the hilum. Consequently, the most marked involvement of the tissues is still found around the large bronchi and vessels near the hilum. Perivascular cuffing, in which lymphocytes are numerous, is conspicuous.

"Minute Bodies."—In stained sections of the consolidated lungs from monkeys, the "minute bodies" found in livers, spleens, etc., of parrots and mice infected with the virus of psittacosis were searched for diligently. None were seen. Failure to find them, however, does not necessarily indicate that they were not present in small numbers. Many cells in the alveoli, alveolar walls, and perivascular cuffs contained granules of different sizes and nature. None of these however, resembled the "minute bodies."

Psittacosis Pneumonia in Rabbits

After we recognized the fact that intracerebral inoculations of the virus do not induce pneumonia in monkeys, while intratracheal and intranasal injections of the same agent do, we decided, in spite of our previous negative results with intracerebral methods, to infect some rabbits intratracheally in order to ascertain whether a pneumonia can be produced in these animals by such a procedure.

2 groups of rabbits were used. One set of animals received intratracheally 1 cc. each of an emulsion of livers and spleens from mice infected with psittacosis virus, the rabbits of the other group were similarly inoculated with an emulsion of livers and spleens from normal mice. The animals that received the infectious material developed fever. Some of them died, while others were sacrificed at different intervals for bacteriological and pathological studies. Cultures of the lungs for the

presence of aerobic and anaerobic bacteria remained sterile. Macroscopically and microscopically the lungs showed a pneumonia similar to that observed in monkeys 2-4 days after intratracheal injections of the virus. The rabbits that received the emulsions of normal livers and spleens had no fever and, when they were sacrificed for examination of their lungs, revealed no pneumonia.

From the results of the above experiment it is obvious that rabbits develop pulmonary lesions following intratracheal injections of psittacosis virus. Experiments reported in the third paper of this series, however, show conclusively that such lesions do not occur, or only rarely, when the infectious agent is placed intracerebrally.

DISCUSSION

The evidence—clinical findings, X-ray photographs, gross and microscopic pathology, and bacteriological observations—justifies the conclusion that emulsions of livers and spleens from psittacosis-infected mice injected intratracheally or instilled intranasally in monkeys are capable of producing a pneumonia unassociated with ordinary bacteria. Moreover, such a pneumonia is similar to, if not entirely identical with that observed in man (1-4) infected with the virus of psittacosis. Furthermore, the experimental study of the disease in monkeys offers opportunities for observation that cannot be made in man, because the animals can be sacrificed at any time during the course of the malady and in them pictures uncomplicated by secondary bacterial invasion are the rule rather than the exception.

The pneumonia occurring in monkeys under experimental conditions begins around the large bronchi and vessels near the hilum and apparently spreads towards the periphery along the alveolar walls. Resolution occurs in the reverse order. The pleura is rarely involved. Many of the evidences of pulmonary involvement—vascular engorgement, cellular infiltration, necrosis of alveolar walls, hemorrhage, serous exudation, fibrin deposition, desquamation of alveolar epithelium, distention of alveoli by polymorphonuclear and mononuclear cells—observed in the psittacosis-infected lungs have been described in other infectious processes of this organ. Yet the peculiar combination of these pathological processes in lungs infected with psittacosis virus immediately distinguishes the reaction from ordinary pneumonias and from ordinary bacterial infections of the lungs.

From the evidence presented in the three preceding papers it is obvious (1) that parrots, regardless of the portal of entry of the inciting agent, rarely, if ever, develop a pneumonia as a result of an infection with the virus of psittacosis, (2) that rabbits and guinea pigs do not have an involvement of the lungs when inoculated intracerebrally, intradermally, or intraperitoneally, (3) that mice inoculated intracerebrally or intraperitoneally remain free from pulmonary lesions. Furthermore, in the present paper it has been shown that monkeys receiving the virus in the brain, although they become infected, develop no pneumonia. Opposed to such observations are those whereby it has been demonstrated that rabbits and monkeys, the former inoculated intratracheally, the latter infected either intratracheally or intranasally, exhibit pulmonary lesions. From these observations one is justified in surmising that psittacosis pneumonia in man probably results from the entry of the virus through the upper respiratory tract. Moreover, the results of the work reported in the first and second papers of this series indicate that the source of the virus in parrots and in man is the nasal and oral secretions and feces of the former and sputum of the latter. Thus in the case of man, a fair conception concerning the source of the infection and the portal of entry of the virus has been obtained. Consequently the precautions necessary for prevention of the infection are obvious. Despite them it is difficult, nevertheless, to prevent the spread of the disease from parrots to human beings, as witnessed by laboratory infections occurring under good conditions. Spread of the malady from man to man occurs infrequently, however. Certain possible reasons, such as amount of inoculum encountered or alteration in the virulence of the virus in the human host, may account for the apparent difference in the contagiousness of the disease.

CONCLUSIONS

The virus of psittacosis inoculated intratracheally or intranasally in monkeys produces a pneumonia similar to that caused by the same active agent in man.

Intracerebral inoculation of the virus induces a meningo-encephalitis characterized principally by a mononuclear reaction in the meninges.

Indirect evidence has been adduced to show that the portal of entry of the virus in man is the upper respiratory tract.

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EXPLANATION OF PLATES

PLATE 12

- FIG. 1. Monkey H, 2 days postinoculation. Roentgenogram of chest showing extensive mottling of right lower lobe with smaller shadows in the left lower. There is also evidence of spreading of the infection along the bronchial tree into the upper and middle lobes.
- FIG. 2. Monkey K, roentgenogram of chest before inoculation.
- FIG. 3. Monkey K, 5 days postinoculation. Two shadows at hilum on right side.
- FIG. 4. Monkey J, 6 days postinoculation. Roentgenogram of chest: The right lung is almost completely involved, the left lower seems to be extensively consolidated, the left upper and middle lobes are relatively clear.
- FIG. 5. Monkey I, roentgenogram before inoculation.
- FIGS. 6-10. Monkey I, roentgenograms showing the progression and regression of the consolidation. The pictures were taken 2, 5, 9, 11, and 13 days respectively after inoculation. Note rapid clearing within 48 hours, as evidenced by Figs. 9 and 10.
- FIG. 11. Monkey D, roentgenogram before inoculation.
- FIG. 12. Monkey D, 4 days postinoculation. Roentgenogram of chest showing involvement of a large part of the right lung and of the left lower lobe.

PLATE 13

- FIG. 13. Monkey H was sacrificed 2 days after inoculation. The painting shows a beginning pneumonia near the hilum. $\times 1$.
- FIG. 14. Monkey F died 6 days after inoculation. The painting reveals extensive pneumonia. Yet the edges of the involved lobes are not completely consolidated. Note the peculiar lilac-pink color. $\times 1$.

PLATE 14

- FIG. 15. Monkey H, 2 days postinoculation. Section of lung showing spread of the infection along the alveolar walls. $\times 125$. Eosin and methylene blue.
- FIG. 16. Monkey H, 2 days postinoculation. Engorgement of blood vessels, serous exudate in alveoli, and polymorphonuclear leucocytes. $\times 450$. Eosin and methylene blue.

FIG. 17. Monkey H, 2 days postinoculation. In this part of the consolidated lung, polymorphonuclear leucocytes are absent. Compare with Fig. 18. $\times 450$. Eosin and methylene blue.

FIG. 18. Monkey H, 2 days postinoculation. Polymorphonuclear leucocytes are abundant in this portion of the involved lung. Compare with Fig. 17. $\times 450$. Eosin and methylene blue.

FIG. 19. Monkey K, 7 days postinoculation (intranasal). Note the thick alveolar walls and the large mononuclear cells in the alveoli. Polymorphonuclear cells are absent. $\times 450$. Eosin and methylene blue.

PLATE 15

FIG. 20. Monkey D, 4 days postinoculation. Section reveals different stages in the process of consolidation. The portion of the tissue least involved is near the periphery of the lobe. $\times 125$. Eosin and methylene blue.

FIG. 22. Monkey D, 4 days postinoculation. Alveolar walls are necrotic. Fibrin and many polymorphonuclear cells are present. $\times 450$. Eosin and methylene blue.

FIGS. 21, 23, 24. Monkey D, 4 days postinoculation. The alveolar walls are thickened and the alveoli contain desquamated epithelial cells together with other types of mononuclear elements. Very few polymorphonuclear leucocytes are seen. $\times 450$. Eosin and methylene blue.

PLATE 16

FIG. 25. Monkey A, 8 days postinoculation. The alveolar walls are greatly thickened by cellular proliferation and infiltration. The cells lining the alveoli are swollen and some are seen free in the sacs. $\times 450$. Eosin and methylene blue.

FIG. 26. Monkey A, 8 days postinoculation. The alveolar spaces contain various kinds of mononuclear cells. $\times 450$. Eosin and methylene blue.

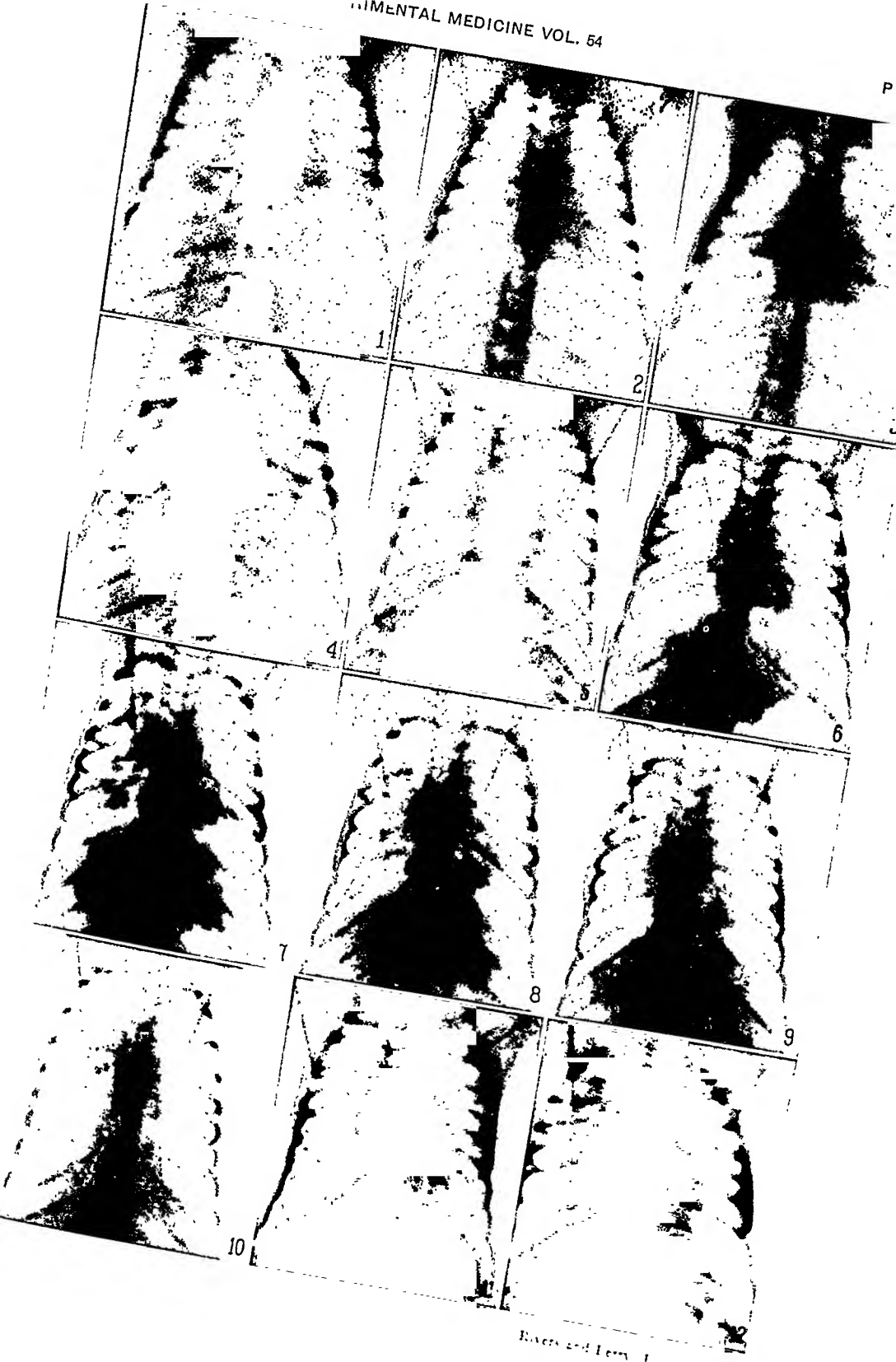
FIG. 27. Monkey A, 8 days postinoculation. It is difficult to distinguish the thickened alveolar walls from the cellular contents of the alveoli. $\times 450$. Eosin and methylene blue.

PLATE 17

FIG. 28. Monkey I, 13 days postinoculation. At the time the monkey was sacrificed, resolution was progressing rapidly. Yet the section seems to indicate that organization had occurred. Compare with Figs. 29 and 30. $\times 450$. Giemsa.

FIG. 29. Monkey I, 13 days postinoculation. Section showing resolving pneumonia. Many alveoli are greatly distended with air, while others still contain exudate. $\times 125$. Aniline blue and orange G.

FIG. 30. Monkey I, 13 days postinoculation. The aniline blue and orange G stain clearly indicates that very little new connective tissue has been laid down. Compare with Figs. 28 and 29. $\times 450$.





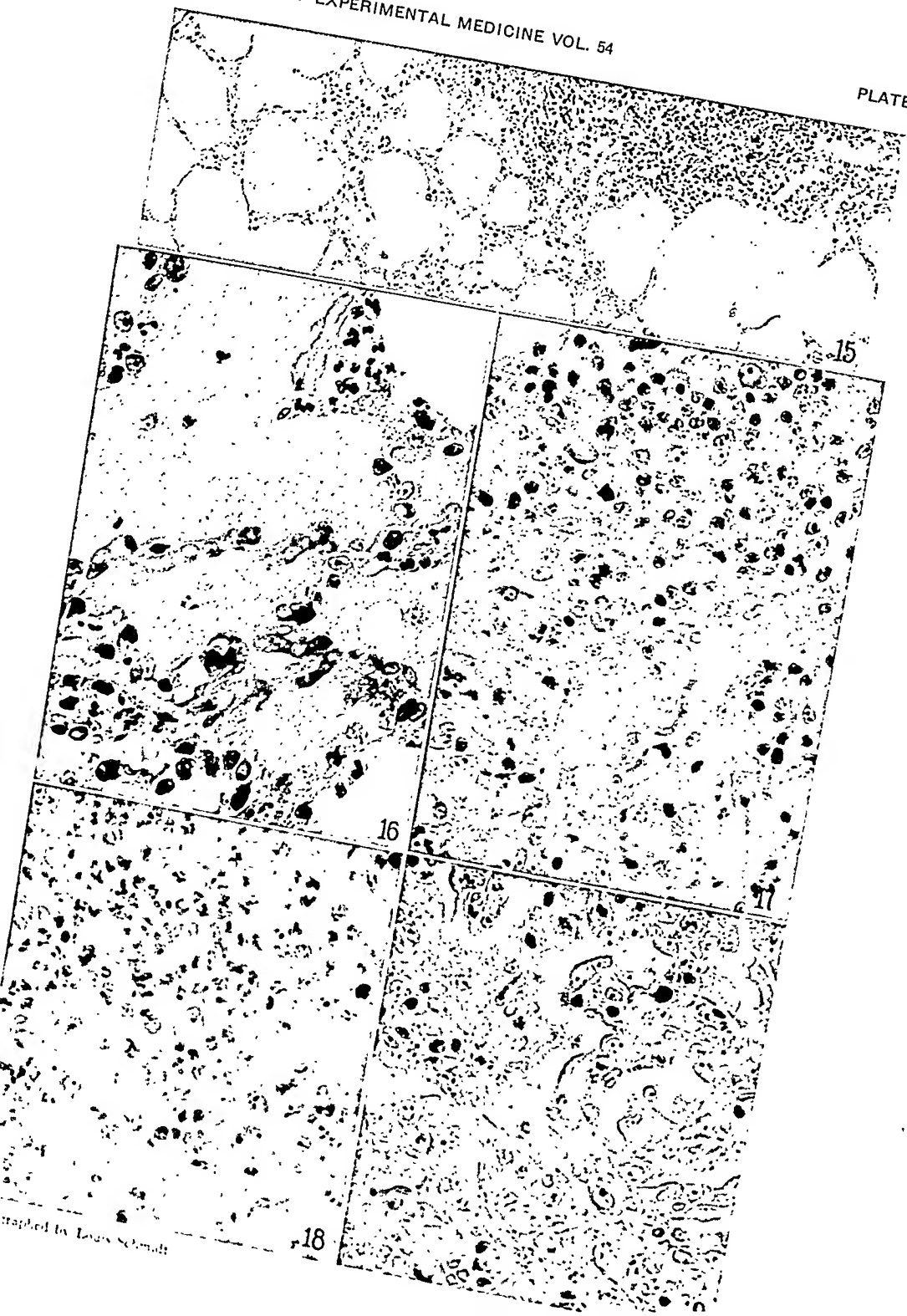
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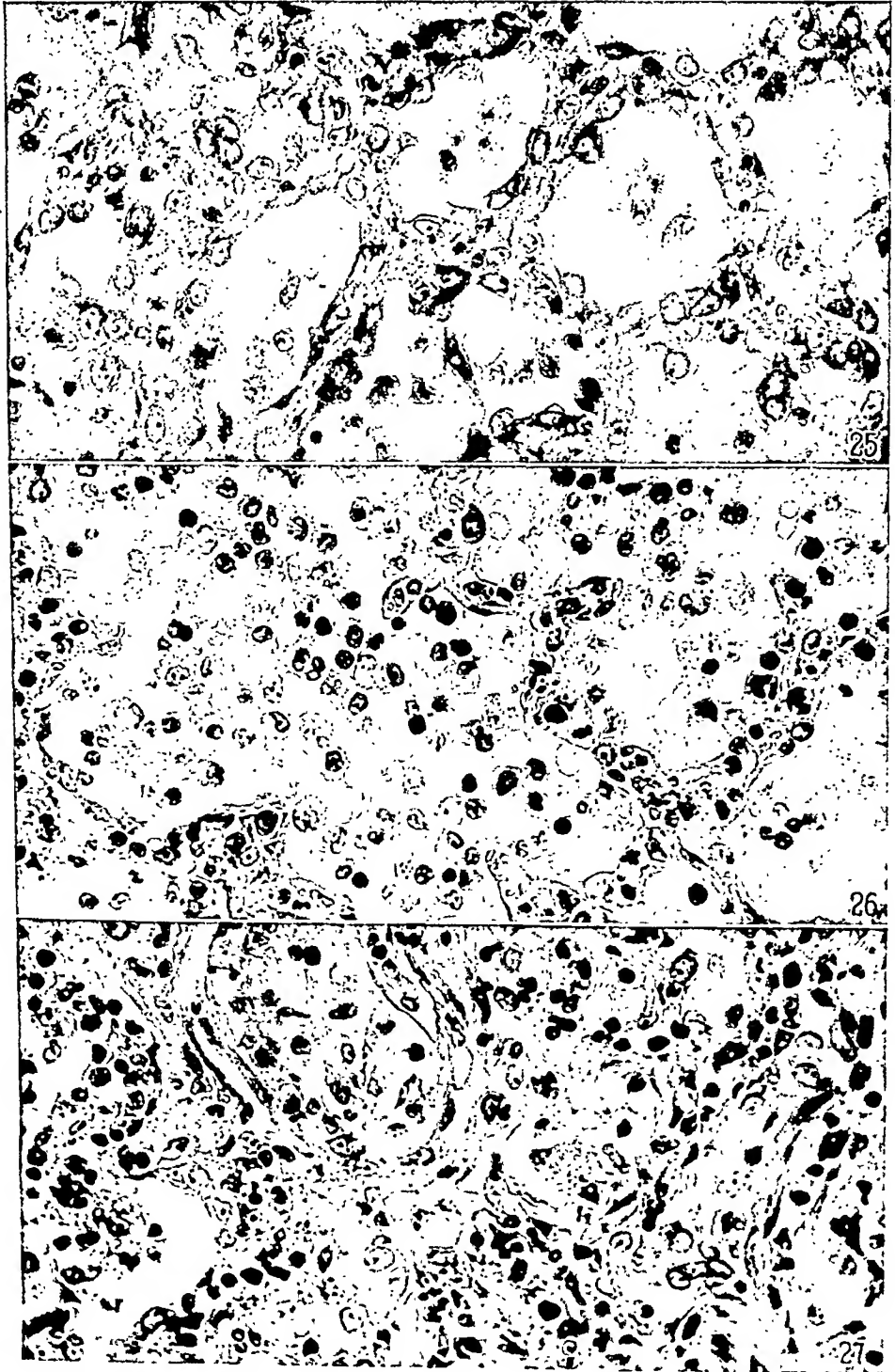
Painted by Louis Schmidt

(River)



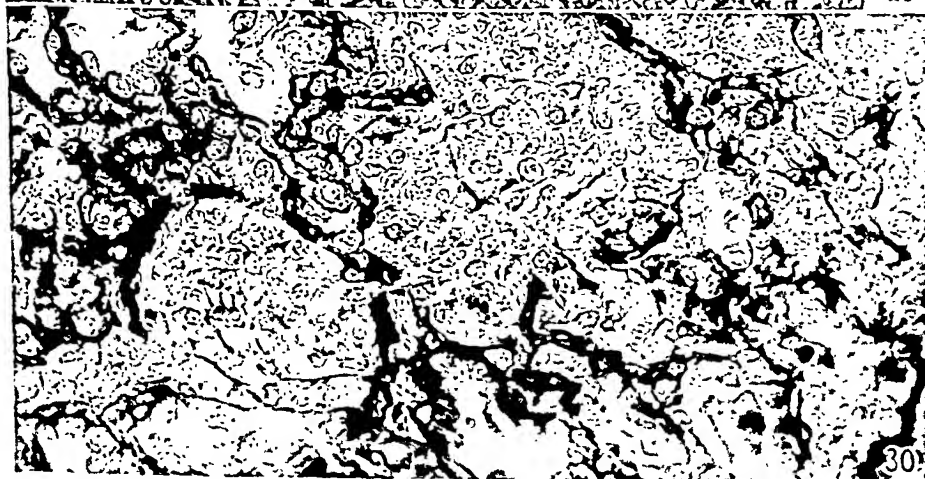
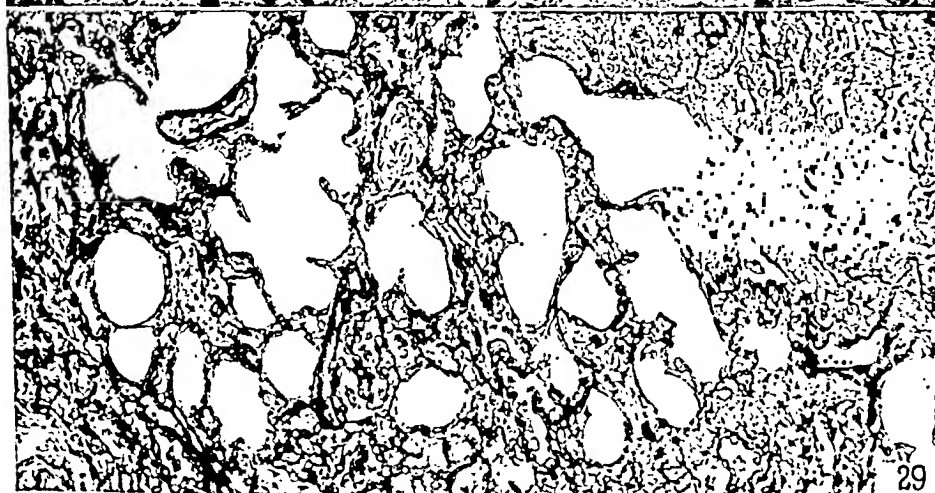
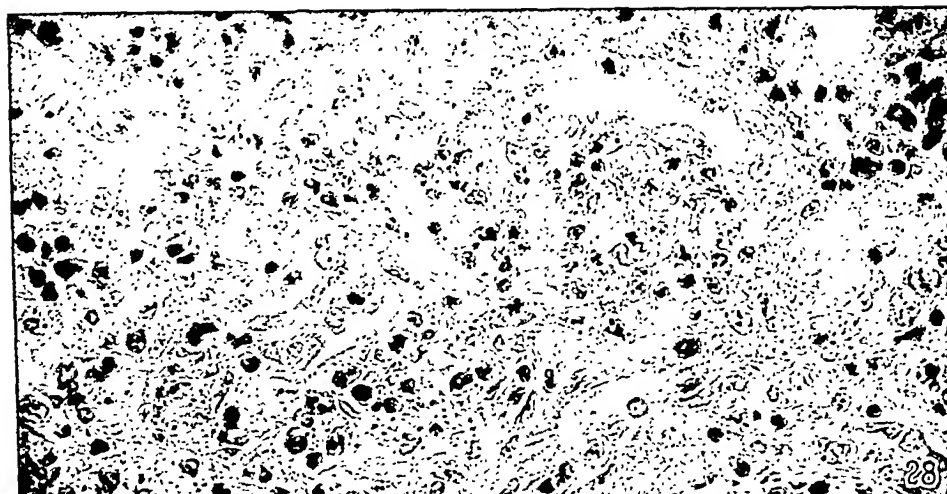


prepared by Louis Schmidt



Photographed by Louis Schmidt





Photographed by Louis Schmidt

(Rivers and Perry: Polytosis, IV)

NUTRITIONAL MUSCULAR DYSTROPHY IN THE GUINEA PIG AND RABBIT^{1,2,3}

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PLATES 18 AND 19

(Received for publication, April 2, 1931)

In the course of experiments planned to study the relationship between vitamin E and reproduction in the guinea pig, it was discovered that the animals did not survive long enough on the chosen diet for breeding experiments to be carried out, and that they developed an extreme degeneration of the voluntary muscles without obvious lesions in the other organs. The diet was one composed of natural foods, in which the vitamin E had been destroyed by treatment with ethereal ferric chloride, according to the method of Waddell and Steenbock (1). However, it was later found that the addition of vitamin E to the ration did not prevent the lesions; nor was the ethereal ferric chloride treatment necessary for their production. Although the factor responsible for the muscular dystrophy has not yet been determined, we have been able to eliminate known causes of degeneration of muscle fibers, such as starvation, infection, and scurvy. A similar disorder was readily induced in rabbits by the use of the same diets, but rats appeared to be insusceptible.

While comparable lesions have been described as accompanying other disorders, a generalized and apparently primary degeneration of the entire voluntary muscular tissue has not hitherto been produced by dietary means.

¹ Some of the data in this paper are taken from a dissertation submitted by Marianne Goettsch in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Columbia University, 1930.

² This work was aided by the Research Grant from the Chemical Foundation to the Department of Biological Chemistry.

³ A preliminary report was presented at the March, 1930, Meeting of the Society for Experimental Biology and Medicine.

Preparation of the Diet

The diet was prepared as follows:

Diet 13

Rolled oats (Quaker).....	355 parts
Wheat bran (Pillsbury).....	180 "
Casein (Merck technical).....	75 "
Lard.....	80 "
Cod liver oil (Mead, Johnson).....	10 "
NaCl.....	10 "
CaCO ₃	15 "

10 gm. of ferric chloride, U.S.P. lump, was taken up in about 125 cc. of ether and a little water, and the solution poured over the above ingredients. The mass was shaken in a closed container and allowed to stand for about half an hour. The contents were emptied upon a tray and the ether allowed to evaporate, usually overnight. Finally there was added:

Skimmed milk powder (Merrell-Soule)—275 parts.

Diet 11 was similar to Diet 13 except that the treatment with ethereal ferric chloride was omitted. The diets were freshly prepared every 2 to 3 days and were always consumed within 7 days. Diet 13 possessed an acrid odor as described by Waddell and Steenbock (1) in their vitamin E-deficient diet.

Each guinea pig was given daily by pipette 3 or 6 cc. of orange juice.

Diet 13 was calculated to contain 3.7 per cent of roughage, to have a nutritive ratio of 1:3.6, with two-thirds of the proteins supplied by milk products, and a calcium-phosphorus ratio of 1.62:1. Vitamins A and D were supplied by the cod liver oil, B and G by the grain and milk products, and C by the orange juice. The vitamin E content was purposely reduced to a minimum. The description of control experiments with Diet 13 carried out upon rats will follow in a later section of the paper.

Effect of Diet 13 upon Guinea Pigs

Guinea pigs that had been born in the laboratory were used in the present experiments, and upon weaning at 21 days, they were given Diet 13 with lettuce *ad lib.* and orange juice. After about 10 days, when they had become more or less accustomed to the new diet, the greens were discontinued. Other groups of guinea pigs were given either Diet 11, or a stock diet of oats, bran, and greens.

The rate of growth for the first month or two was good for all groups. After the animals had attained a weight of about 400 gm., there was a sudden cessation in the growth of the animals on Diet 13; this was accompanied by general flabbiness, and it was observed that the guinea pigs had difficulty in righting themselves if they were placed

on their back. During the following month, a constant weight was usually maintained, after which the guinea pigs went into rapid decline for 2 or 3 days and suddenly died. A few of the animals recovered spontaneously from the flabbiness, made a moderate gain in weight, remained stationary at a higher level, and died. The terminal loss in weight was often as great as 15 to 20 per cent of the maintenance weight.

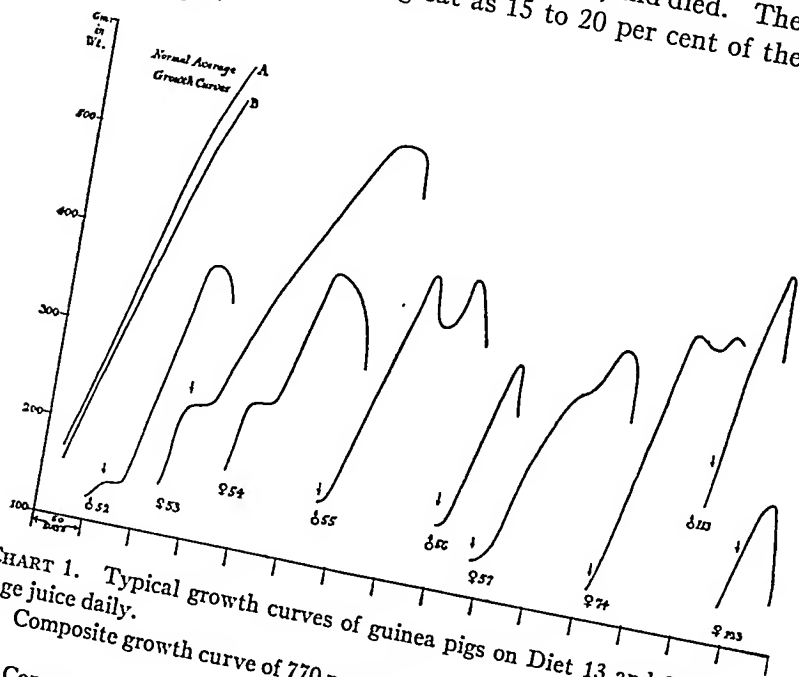


CHART 1. Typical growth curves of guinea pigs on Diet 13 and 3 or 6 cc. of orange juice daily.
A. Composite growth curve of 770 normal male guinea pigs from data of Wright (2).
B. Composite growth curve of three males and four females on the stock diet of

The average survival period was about 90 days but there was great individual variation, as is shown in Table I. Two guinea pigs died after only 35 and 37 days on the diet, while two others lived for 186 and 218 days. Litter mates were found to vary as much as others in this respect. The group on stock diet showed no break in the growth curve and continued to increase in weight steadily. Typical growth curves of guinea pigs on Diet 13 are illustrated in Chart 1. In Chart 2, the growth curve of Female 53 is shown in de-

tail. This guinea pig lived for 186 days on Diet 13. Estrus, indicated by the letter E, is seen to have occurred regularly at the normal intervals of 16 to 18 days, (Stockard and Papanicolaou (3)) even when

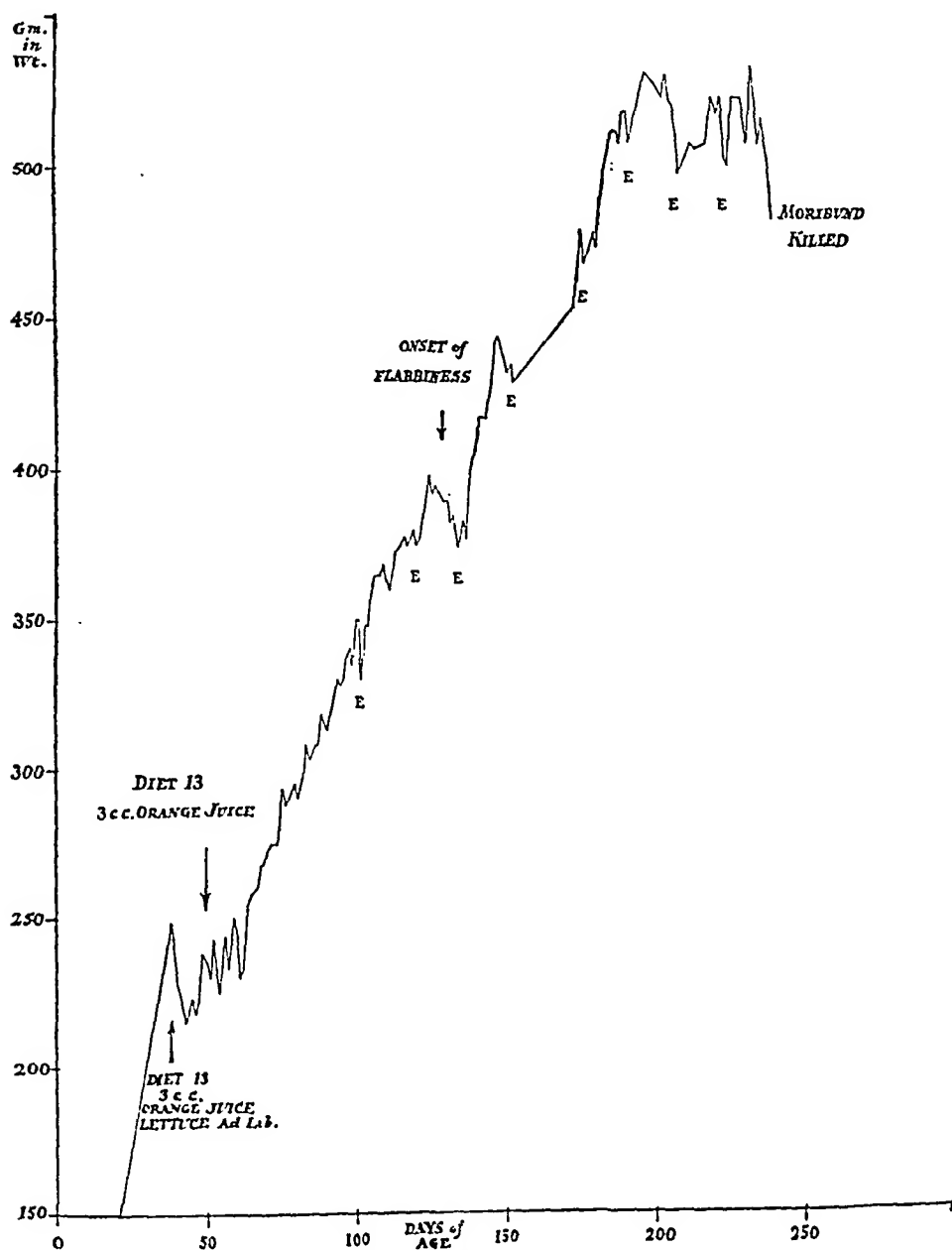


CHART 2. Growth curve and estrous rhythm of Guinea Pig ♀ 53 on Diet 13 and 3 cc. of orange juice daily. Letter E indicates estrus.

the animal had begun to show definite symptoms. The estrous rhythm was generally normal in the guinea pigs on Diet 13 as well as on Diet 11. Further studies upon reproduction were impossible on account of the early death of the animals. Males reared on Diet 13 showed, at autopsy, normal-looking motile sperm in smears from the epididymis, although one had been on the diet as long as 218 days.

Pathological Changes

Gross Appearances.—While most of the animals were killed and autopsied when apparently on the point of death, a few were sacrificed before the cessation of growth. Of the 30 experimental guinea pigs, all but three showed striking macroscopic changes in the skeletal muscles. The muscles of the thigh and abdomen were particularly abnormal, although the muscles of the back and the extremities, the diaphragm, and the intercostal muscles were involved. They were atrophied, pale, with a yellowish or yellowish brown color, and less translucent than those of the controls. Sometimes they appeared gritty and streaked as though calcified or infiltrated with fat. In addition to their abnormal appearance, the muscles had lost their irritability to mechanical stimulation and failed to contract when severed.

In contrast to the wasting of the skeletal muscles, the subcutaneous adipose tissue and that within the peritoneal cavity were often strikingly abundant, an indication that the fall in weight was at the expense of the muscle rather than of the fat. No gross changes of significance were noted in the internal organs. The heart was normal. The lungs in some animals showed areas of pneumonic consolidation, in others were well aerated and of normal appearance. The liver in the majority of animals showed fat infiltration. The spleen was small, firm, and brownish. The kidneys, adrenals, genitalia, and gastrointestinal tract were normal.

Microscopic Lesions.—The pathological changes of significance were restricted to the striated skeletal muscles of the trunk and extremities, in which the lesions were profound, widespread, and characteristic. In guinea pigs, practically every voluntary muscle, including the diaphragm, was affected to a varying degree with the exception of the masseter and tongue muscles, which preserved their integrity to

the end. The muscles examined as routine were the quadriceps femoris, the adductors of the thigh, the pectorals, the abdominal muscles, the diaphragm, and the masseter. At various times, blocks were taken from many other muscles as well.

The histological changes depend somewhat upon the stage of the disease, but the essential and primary lesion is a coagulative necrosis of the muscle fiber, presenting the familiar picture of waxy, hyaline, or Zenker's degeneration (Fig. 1). This is followed by reactive cellular multiplication with attempts at regeneration. Many of the necrotic fibers become resorbed and their place taken by fat and connective tissue. The late stages, as will be shown, resemble closely the changes seen in progressive muscular dystrophy of man.

While all the muscles examined showed these degenerative lesions to a greater or lesser degree, the muscles were not always uniformly affected, certain bundles being but slightly involved, while in others almost 100 per cent of the fibers were necrotic.

The earliest change in the fiber is shown in the appearance of transverse ridges or contraction bands, in which the striations are brought more closely together. The discs become disarranged and lose their individual identity to fuse into a swollen hyaline mass. Fibers cut in their long axis may show a succession of more or less globular hyaline masses (Fig. 1), between which the sarcolemma is collapsed, containing only granular detritus. The sarcolemma nuclei are displaced and distorted, and tend to become oriented at right angles to the long axis of the fiber; subsequently they undergo karyorrhexis and may disappear completely.

Preparations from many of the animals were obtained in this stage, before any marked cellular reaction had occurred. In most instances, however, side by side with the necrobiotic changes, there was found an active multiplication of cellular elements with numerous mitoses. The cells have deeply staining oval nuclei, and a rather small amount of purplish cytoplasm. They lie in clefts in the coagulated and necrotic muscle substance, and may completely fill the original sarcolemma sheath. The cylindrical columns of closely packed cells constitute the "*Muskelzellenschläuche*" of Waldeyer (Fig. 2). The origin of these cells, whether from invading histiocytes or from the division of the uninjured muscle nuclei with their surrounding sarcoplasm, has been often discussed. Reference may be made to the excellent studies of Forbus (4), who by means of preliminary vital staining, was able to distinguish the invading phagocytic histiocytes, which aid presumably in the removal of the necrotic material, from the proliferating muscle nuclei, which are concerned in the regeneration of new fibers.

It has seemed to us that the majority of the mononuclear cells making up the "*Muskelzellenschläuche*" were derivatives of the muscle cells themselves, although we have occasionally observed the invasion of the dead fibers by polymorphonuclear leucocytes (Fig. 3). Many of them, as Forbus and others have pointed out, undergo subsequent degeneration, but others assume a spindle shape, align them-

selves in rows, and by the development of myofibrils on their surface, give origin to regenerating muscle fibers (Fig. 4). Because of their basophilic staining, such young regenerating fibers are easily distinguished from the persistent original fibers which have escaped destruction. In many of our preparations, there is a conspicuous formation of large multinucleated plasmatic masses, lying against the necrotic remains of the muscle substance (Fig. 5). Calcification of fibers was occasionally seen (Fig. 6).

It has been stated that in the more chronic cases, only occasional necrotic fibers were to be seen, but the remaining fibers were often atrophic and widely separated by strands of cellular connective tissue or by fat. These muscles with replacement fibrosis and lipomatosis closely resemble those of progressive muscular dystrophy in man (Fig. 7).

The pathological changes described above are definitely restricted to the striated skeletal muscles. The myocardium and the smooth muscle fibers of the intestine, bronchi, blood vessels, and uterus are entirely unaffected.

As regards the question whether the degeneration of the muscles is primary, or secondary to alterations in the nervous system, it may be said that the spinal cord and peripheral nerves show no significant changes. While the nervous tissues have not been systematically examined in all cases, sections have been made in sufficient number to show clearly that the disease of the muscles is not associated with degeneration of the ganglion cells of the central nervous system nor of the peripheral nerve trunks. Even in muscles showing extreme destruction of the great majority of the fibers, the small intermuscular nerves present no recognizable alteration.⁴ There still remains the possibility that the first injury falls upon the motor end plates; this needs further investigation.

That the muscle lesions may develop very rapidly is indicated by the following observations:

Guinea Pig 101, male. Born Dec. 16, 1929. Weaned Jan. 12, 1930. Diet 13 plus 6 cc. of orange juice started on Jan. 22, 1930. On Mar. 24th, after having been 61 days on the diet, a piece of adductor muscle was excised from the right high. Two blocks examined showed practically no abnormal fibers: near the edge

⁴ We are indebted to Dr. W. M. Rogers of the Department of Anatomy and to r. Abner Wolf of the Department of Pathology for silver preparations of our material which demonstrate the integrity of the terminal nerves in severely degenerated muscles.

of the section was a single muscle cell column. On the 75th day, at which time the decline in weight had begun and distinct symptoms of muscular weakness had become manifest, another muscle specimen was excised from the left adductor. The lesions were now marked: there were numerous necrotic fibers and much cell proliferation.

The animal died on the following day, and was found to have most intense lesions of all the muscles examined.

In this animal, therefore, there is evidence that the alterations in the muscle lesions occurred only during the last 2 weeks of life.

In several other animals, the progress of the disease was followed by removing pieces of muscle for microscopic study at various times after beginning the experimental diet. The rather abrupt onset of the muscle lesions was again noted. Thus in Guinea Pig 107, specimens obtained on the 36th and 49th days showed no lesions. On the 65th day, there were occasional necrotic fibers, with moderate multiplication of the muscle nuclei. On the 78th day, the lesions were very marked. There were numerous necrotic and segmented fibers, and great proliferation of the muscle nuclei. The animal died on the 108th day and showed advanced changes, with much fibrous and fatty replacement of the degenerating muscles.

Microscopic Changes in Other Organs and Tissues.—The myocardium was unaltered. The lungs, in a large proportion of the guinea pigs and rabbits were the seat of a bronchopneumonia of varying extent, often associated with areas of atelectasis. In a number of the animals, the lungs were normal. The liver frequently exhibited a moderate degree of fat infiltration. The spleen was small and brownish; it contained hemosiderin deposits in the pulp in definitely increased amount.

None of the other viscera, including pancreas, adrenals, kidneys, gonads showed changes to which any importance could be attached. As might be anticipated, the deficiency in vitamin E did not inhibit spermatogenesis, the duration of the survival period being too short for this.

Summary

A general oversight of our material is given in Table I. Of thirty guinea pigs on Diet 13, eight were not studied microscopically; however, six of these presented the symptoms and gross changes which were later recognized as characteristic and the remaining two manifested symptoms, but no gross lesions were noted. The remaining twenty-two showed characteristic microscopic changes.

Effect of Diet 13 upon Rabbits

Two rabbits, Nos. 1 and 3, born in the laboratory, were placed upon Diet 13 with 6 cc. of orange juice daily, when they were 4½ weeks of age. They maintained

TABLE I
Showing the Effect of Diet 13 upon Guinea Pigs

Guinea pig	Days on diet	Extent of muscle lesion		Lung infection	Remarks
		Gross	Microscopic		
♂ 52	76	Normal	Not examined	+++	Moribund, killed
♀ 53	186	++++	++++	+	" "
♀ 54	97	++	++	Normal	" "
♂ 55	119	++	Not examined	+	" "
♀ 56	48	++	" "	Normal	" "
♂ 57	122	++	++	+	" "
♀ 58	122	++	+++	Normal	" "
♂ 59	133	++	+++	+	" "
♀ 60	77	+++	Not examined	Normal	" "
♀ 61	110	++	" "	+++	" "
♀ 62	134	++	++++	+	" "
♀ 63	92	+++	Not examined	Normal	Died
♀ 68	63	+++	+++	+	Moribund, killed
♀ 72	72	+++	+++	Normal	Died
♀ 74	98	+	++	?	" , advanced p.m. changes
♀ 80	58	+++	+++	+	Moribund, killed
♀ 81	55	++	++++	Normal	Biopsy
♀ 85*	58	++	+++	+	Moribund, killed
♂ 86*	35	+	+++	+	" "
♂ 93	40	Normal	+++	Normal	" "
♂ 101	77	+++	Not examined	+	Died, advanced p.m. changes
♂ 104	24	+	+++	Normal	" , advanced p.m. changes
♂ 107	85	+	Not examined	+	" "
♂ 109	218	++++	+++	Normal	Moribund, killed
♀ 110	131	+	++++	+	Died, advanced p.m. changes
♀ 113	51	+++	+++	Normal	" "
♀ 115	137	+++	+++	+	Moribund, killed
♂ 116	76	++	++	Normal	Died, advanced p.m. changes
♂ 119	133	++	+++	+	Moribund, killed
♂ 123	37	+++	+++	+	" "
			Normal	+	" "
			Normal	+	" "

* Whole wheat substituted for bran in Diet 13.

their weight and even grew to some extent for 12 days, then lost the use of their muscles completely, and were killed and autopsied 2 and 4 days later. The muscles

were small, extremely pale, and without irritability. Five older rabbits, weighing from 385 to 730 gm., were also given Diet 13 with orange juice. They survived for a longer time, but after 24 to 50 days became prostrated and upon autopsy showed marked macroscopic changes in the voluntary muscles. The other organs apparently were normal.

The muscle lesions in rabbits are essentially the same as those in guinea pigs. They are in general more rapid in their development; extreme lesions have been noted as early as 15 days after the institution of the diet. The masseter shares in the general muscular degeneration, and there is greater tendency for the necrotic fibers to become

TABLE II
Showing the Effect of Diet 13 upon Rabbits

Rabbit	Weight at beginning of experiment	Days on diet	Extent of muscle lesion		Lung infection	Remarks
			Gross	Microscopic		
♀ 1	335	14	+++	++++	+	Moribund, killed
♀ 3	340	16	+++	++++	+	" "
4	450	5	Normal	+	Normal	Died
5	425	24	++	+++	+++	Moribund, killed
♂ 10	730	50	++	++	++	Died
11	490	27	Normal	Slides lost	+++	Moribund, killed
12	385	27	+	+++	+++	Died

calcified. We have noted no other differences in reaction between rabbits and guinea pigs.

The experiments with rabbits are summarized in Table II.

Failure of Rats to Develop Muscular Dystrophy on Diet 13

Rats reared on Diet 13 gave no symptoms of the muscular dystrophy characteristic of guinea pigs and rabbits, although they were maintained on the diet for more than a year. Some of the young from mothers on Diet 13 were continued on the diet until they were old enough to be bred. They are listed as second generation in Table III. Young from second generation rats were likewise continued on the diet and formed the third generation. A detailed microscopic study of muscles from a second generation rat, killed at 30 days, revealed an

occasional necrotic fiber, while three stock controls of the same age were normal. Two third generation rats, young from B361 of Table III, were killed at 4 months and were found to have muscles that were entirely normal.

Discussion of Possible Factors

Before attributing these widespread changes in the muscular system to any specific characteristics in the diet, one should exclude, if possible, certain well recognized causes of muscular degeneration and these may be briefly discussed.

1. *Starvation*.—In the terminal stage of the muscular dystrophy there is, as has been shown, a rapid loss of weight. With the increasing muscular paresis, there is often inability to take food. It has been shown by Kremer (5), that long continued fasting in the hibernating frog leads to extreme muscular necrobiosis, associated with excessive multiplication of muscle nuclei. The illustrations in Kremer's article can be duplicated in our material. While a few days of partial inanition should not be expected to result in comparable change, it was thought wise to control this possibility by a limited number of starvation experiments. An adult guinea pig, No. 1, was given only water and 6 cc. of orange juice daily for a period of 12 days, during which the weight declined from 1060 to 840 gm. Sections of masseter, quadriceps femoris, pectoralis, diaphragm, and adductors of the thigh showed complete absence of degenerative or necrobiotic changes. Guinea Pig 31 was fasted for 11 days under similar conditions, the weight declining from 880 to 715 gm. Again no significant changes were found in the voluntary muscles.

It seems evident, then, that in spite of the rapid loss of weight, amounting to 18 and 21 per cent of the original body weight, the muscular lesions cannot be ascribed merely to inanition. The preservation of the adipose tissue even in cases showing extreme muscular degeneration is additional evidence that we are not dealing merely with the effect of inanition.

2. *Infection*.—All the rabbits and half the guinea pigs died with a terminal bronchopneumonia. Whether the powdery diet, unnatural for animals normally subsisting on coarse vegetable food, was a contributory factor, or whether the weakness of the diaphragm and intercostals also predisposed, cannot be stated. Zenker's degeneration, especially of the rectus abdominis, but also of other muscles, is a common accompaniment of typhoid fever, pneumonia, and influenza in man. The question therefore must be answered as to whether the muscle changes in the experimental animals were related to the pneumonia or other terminal infection. This possibility can be very definitely excluded. Many of the guinea pigs in this and subsequent experiments (see Table I) and Rabbits 15 (Table IV) and 19

(Table V) died with normal lungs, and without any indication of infection in other organs. Pieces of muscle have been excised and found to show lesions at a time when there was little or no loss of weight or other evidence of illness. Furthermore, we have studied as control, muscles from several rabbits with experimental pneumococcus pneumonia, or with general pneumococcus septicemia,⁵ and have found no similar lesions.

3. *Scurvy*.—Inasmuch as several workers, notably Höjer (6), Meyer and McCormick (7), Dalldorf (8) have reported the occurrence of hyaline necrosis of the muscle fibers in the experimental scurvy of guinea pigs, it seemed highly important to be certain that our animals were not suffering from latent scurvy. Sherman (9), using as criterion of scurvy looseness of teeth, fragility of bones, enlargements and hemorrhages of joints and rib junctions, obtained complete protection with a daily addition of 1.5 cc. of orange juice for a period of 90 days. Höjer (10) devised a method for assaying vitamin C based upon the microscopic alterations in the odontoblasts and dentine of the incisor root after 10 to 14 days on the diet. Goettsch and Key (11), comparing this test with Sherman's, found that 3 cc. of orange juice daily was needed for complete protection, and Eddy (12), using the same technique, arrived at a similar quantitative result. Dalldorf and Zall (13) found slight scorbutic lesions in the incisor roots with 5 cc. Using the rate of tooth growth as a test, they found that 5 cc. was not quite adequate, and that "for short periods and conservative diagnosis, 7 cc. might be considered protective."⁶

On the basis of the evidence of previous workers, it might be assumed that the addition of 3 or 6 cc. of orange juice daily should protect against the gross manifestations of scurvy, although it might not give complete protection if the microscopic alterations of the teeth or their rate of growth are taken as adequate criteria. The generally recognized evidences of scurvy were not present in our animals. Hemorrhages were never seen, either in the vicinity of the joints or elsewhere, even in animals showing the most advanced and universal muscle changes. Microscopic section of the ribs showed not the slightest evidence of scurvy, osteogenesis being active, the marrow free from fibrotic changes, and hemorrhages absent. While the teeth have not been studied in all cases, sections from animals with advanced muscular lesions have shown in a number of instances (Fig. 8), normal incisor roots.⁷ Finally, the production of identical muscular changes in rabbits, which are notoriously resistant to experimental scurvy, makes it highly improbable that the muscular lesions are due to a deficiency in vitamin C.

4. *Possible Deficiencies of Diet 13 in Other Vitamins. Vitamin A*.—That the vitamin A content of Diet 13 was not destroyed by the ethereal ferric chloride

⁵ We are indebted to Mrs. Frances M. Weld for placing these animals at our disposal.

⁶ Personal communication from Dr. Dalldorf.

⁷ We wish to express our thanks to Professor Charles Bödecker of the School of Dentistry, for preparing sections of the teeth.

treatment, was proved: first, by the fact that histological sections of epithelial tissue from guinea pigs reared on the diet revealed none of the keratinization described by Mori (14) and Wolbach and Howe (15), as typical of vitamin A deficiency; and secondly, by the failure of rats reared on the diet to show in their estrous cycles, the prolonged period of "cornified" cells shown by Evans and Bishop (16) to be associated with lack of vitamin A.

TABLE III
The Effect of Diet 13 on Female Rats

Showing the occurrence of resorptions, typical of vitamin E deficiency, and their cure by the administration of substances containing E.

Rat	Generation	1st gestation		2nd gestation		Gestation following resorption		
		R.B.C.*	No. of young	R.B.C.	No. of young	Curative substance	R.B.C.	No. of young
W47	1st	+						
W57	1st	+	5					
W64	1st	+	0	+	0			
W65	1st	+	2					
W66	1st	+	6	+	0			
W233	1st	+	2	+	0	Diet 11		
W281	1st	+	1 (dead)	+	0	" 11	+	8
BH298	1st	+	5	+	0	—	+	9
W350	1st	+	6	+	0	Diet 11		
B361	1st	+	0	+	4 (2 dead)	—	+	9
B362	2nd	+	5		0			
B363	2nd	+	5	+	0	550 mg. W.G.O.	+	0
B364	2nd	+	4 (1 dead)	+	0	550 "	+	9
B365	2nd	Never mated		+	0	550 "	+	0
W366	2nd	"	0		0	550 "	+	5
		+				—	+	5

* R.B.C. + indicates successful implantation.

Vitamin D.—That there was sufficient vitamin D in the diet was indicated by the fact that neither rabbits nor rats, including second generation animals, showed rickets in the course of these experiments. Rickets has never been observed in guinea pigs.

Vitamins B and G.—The amount of vitamins B and G necessary for guinea pigs and rabbits is not known. However, since the diet contained an adequate amount of B and G for the rat, and since the guinea pigs and rabbits were free from any evidences of polycneuritis or dermatitis, it seems improbable that a deficiency in these factors entered into the problem.

The Deficiency in Vitamin E

That Diet 13 was deficient in vitamin E content, as planned, was proved by the following experiments with rats:

Rats both male and female, when reared upon Diet 13, responded to the technique of Evans and Burr (17) in a way typical of vitamin E deficiency. Five males after an initial period of fertility, were unable to sire litters after 200 to 250 days on the diet. Upon autopsy, after 230 days, two functionally sterile males showed, in smears from the epididymis, histologically normal spermatozoa, a few of which were motile. In smears from the other three males after 420 days on the diet, there were found either no sperm at all, or many dead atypical ones. Three litter mate control rats upon untreated Diet 11 retained their fertility as judged by functional tests, and at 420 days had normal motile sperm.

Table III shows the reproductive history of thirteen female rats on Diet 13, that were bred at the age of 3 months. Three rats had typical resorption of the embryos during the first gestation, nine had "first litters" followed by resorptions in subsequent pregnancies, and one had two litters. Following resorptions, cures were attempted with Diet 11 as well as with a known source of vitamin E, wheat germ oil. As is shown in Table III, three females were successfully cured by giving them Diet 11 throughout the trial gestation period and three of five females were cured by the administration of 550 mg. of wheat germ oil on the day of positive mating.

Thirty young rats from mothers that had been reared and maintained on Diet 13 during gestation and lactation, did not suffer from the paralysis sometimes shown by young from mothers on a vitamin E-deficient diet during lactation (Evans and Burr (18)).

Elimination of Vitamin E as a Factor in the Production of Muscular Dystrophy

Since Diet 13 was originally planned and subsequently proved to be deficient in vitamin E, it was necessary to ascertain whether the muscle lesions in guinea pigs and rabbits might be a hitherto unrecognized effect of a deprivation in this factor. This question was approached in three ways: (1) by supplementing Diet 13 with vitamin E in the form of tested wheat germ oil, (2) by placing the animals on a vitamin E-low diet made up of simplified foods, such as was used by Evans and Bishop in their experiments upon rats, and (3) by giving the Diet 13 without ferric chloride treatment (Diet 11). The last experiment also served to test the possibility that the ferric chloride *per se* had a deleterious effect upon the muscle, or that it acted destructively upon some unknown necessary constituent in the experi-

mental diet, or that its presence in the diet resulted in the formation of some substance toxic for the muscle tissue.

1. *Addition of Wheat Germ Oil to Diet 13.*—Ten guinea pigs were given Diet 13 plus a daily supplement, administered by pipette, of 6 cc. of orange juice and 8 drops (approximately 200 mg.) of wheat germ oil. This wheat germ oil was assayed for vitamin E content according to the technique of Evans and Burr (17). When given on the day of positive mating, in a single dose of 550 mg. (a quantity equivalent to 1 drop of oil a day for 22 days), it usually cured female

TABLE IV
Showing the Effect of the Addition of Wheat Germ Oil to Diet 13 upon Guinea Pigs and Rabbits

No. of animal	Weight at beginning of experiment	Daily dose of W.G.O.	Days on diet	Extent of muscle lesion		Lung infection	Remarks
				Gross	Microscopic		
G. P. ♀ 94							
G. P. ♂ 114		200	31	Normal	Normal	+++	Killed
G. P. ♂ 106		200	35	+	"	+	Moribund, killed
G. P. ♀ 111		200	46	Normal	"	+	Died
G. P. ♀ 130		200	50	"	"	Normal	Moribund, killed
G. P. ♀ 103		200	48	+++	++++	"	"
G. P. ♂ 108		200	59	Normal	±	"	Died
G. P. ♀ 97		200	75	"	±	"	"
G. P. ♀ 96		200	122	"	++	"	"
G. P. ♀ 112		200	139	+	+++	+	"
R. 6	415	200	233	+	++	Normal	Moribund, killed
R. 8	420	450	45	++	+++	+++	Died
R. ♂ 13	630	450	32	+	+++	+++	Moribund, killed
R. ♀ 14	540	450	56	+++	+++	+	"
R. ♂ 15	490	450	53	+++	+++	Normal	"
			55	+++	+++		"

rats that had been proved to have resorptions. Cures were invariably obtained with single doses of 1.1 gm. (an amount equal to 2 drops of oil a day). The guinea pigs, therefore, received from four to eight times as much wheat germ oil daily as was found necessary to cure the rats, although their body weight was only two to three times as great; and furthermore, the oil was administered from the day of weaning, so that the total amount given was in considerable excess of that required by rats.

As is shown in Table IV, of the ten guinea pigs on the vitamin supplemented Diet 13, four did not survive very long and showed normal muscles at death.

Guinea Pigs 103 and 108, dying on the 59th and 75th days, had in sections, occasional necrotic fibers with little cellular reaction. The other four guinea pigs, which lived from 48 to 233 days, presented very striking and widespread muscular lesions.

Chart 3 illustrates not only the similarity of the growth curves of animals of this group to those of animals showing characteristic muscle dystrophy on Diet 13, but also the great individual variation among them.

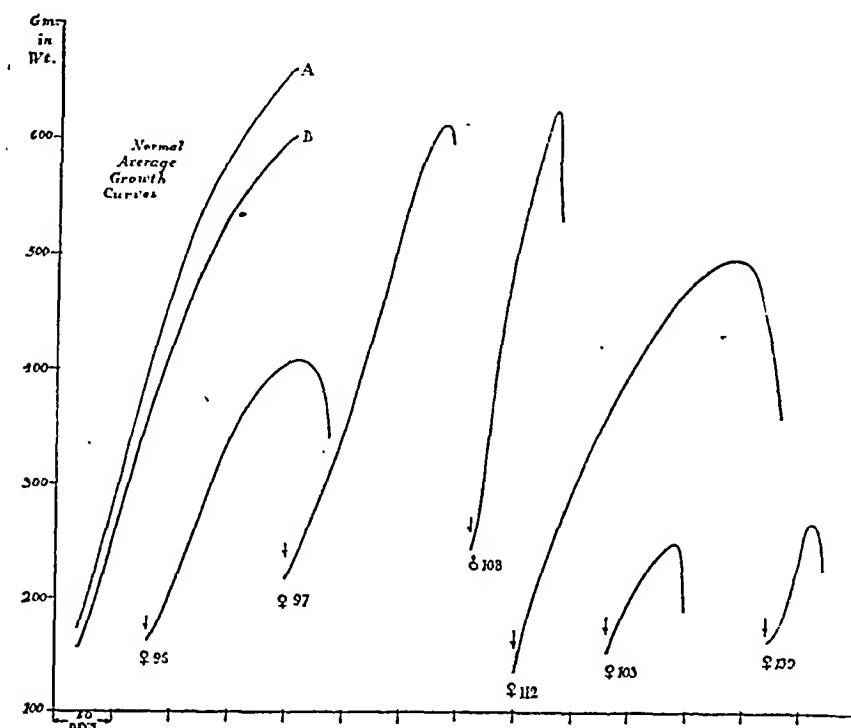


CHART 3. Growth curves of guinea pigs on Diet 13 with the daily supplement of 6 cc. of orange juice and 200 mg. (8 drops) of wheat germ oil.

A. Composite growth of 770 normal male guinea pigs from data of Wright (2).

B. Composite growth curve of three males and four females on the stock diet of oats, bran, and greens.

Table IV also includes data from five rabbits on Diet 13 plus 6 cc. of orange juice and 450 mg. of wheat germ oil. Without exception, they exhibited characteristic symptoms and severe gross and microscopic lesions in the muscles, in the same length of time as the group without the wheat germ oil supplement.

2. *Effect of a Simplified Food Diet with and without Vitamin E upon Guinea Pigs.*—An attempt was made to study the possible rôle of vitamin E deficiency in the development of the muscular dystrophy by placing guinea pigs upon a simplified diet deficient in this factor. The diet consisted of 27 per cent casein, 59 per cent

corn-starch, 8 per cent lard, 4 per cent McCollum salt Mixture 185 (19), 8 per cent Fleischmann's bakers' dried yeast, and 2 per cent cod liver oil, and was found to be adequate for rats except in its vitamin E content.

When this diet with 6 cc. of orange juice was given to fourteen young guinea pigs, half of them altogether refused to eat it, and the other seven made very poor gains in weight and were in an evident state of malnutrition. Five survived for only 11 to 45 days and had normal muscles at autopsy. Of the remaining two, No. 89 lived for 139 days. It never exceeded a weight of 275 gm. At autopsy

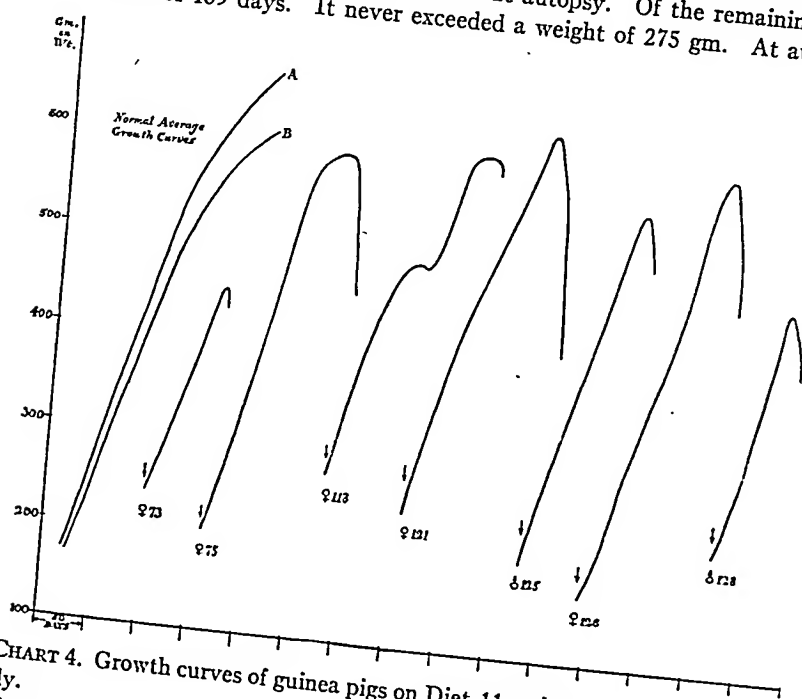


CHART 4. Growth curves of guinea pigs on Diet 11 and 3 or 6 cc. of orange juice daily.

A. Composite growth curve of 770 normal male guinea pigs from data of Wright (2).

B. Composite growth curve of three males and four females on the stock diet of oats, bran, and greens.

the muscles were reduced in bulk, red, dry, and not very contractile. Microscopically there was great atrophy with fibrous replacement; degenerating fibers were still numerous. The masseter as usual was relatively normal. The picture was identical with that seen on Diet 13, when the animal survived for a corresponding period. The other animal, No. 92, had been given 200 mg. of wheat germ oil in addition to the simplified diet and orange juice. It survived for 318 days, reaching a maximum weight of 440 gm. The muscles at autopsy showed only occasional necrotic and calcified fibers.

The unsuitability of this diet for maintaining the guinea pigs in a satisfactory state of nutrition did not justify further experimentation.

3. *Effect of Untreated Diet 11 upon Guinea Pigs and Rabbits.*—Litter mates of the guinea pigs on Diet 13 were maintained on the untreated Diet 11 with 3 or 6 cc. of orange juice a day. As shown in Chart 4, they grew at a normal rate for a longer period of time than the animals on Diet 13, most of them attaining a weight of 550 gm. In the earlier experiments, the controls were actively growing and apparently in excellent condition at the time that their litter mates were dying

TABLE V

Showing the Effect of Diet 11 upon Guinea Pigs and Rabbits

No. of animal	Weight at beginning of experiment	Days on diet	Extent of muscle lesion		Lung infection	Remarks
			Gross	Microscopic		
	gm.					
G. P. ♂ 84*		38	Normal	Normal	?	Biopsy
G. P. ♂ 79		58	"	Not examined	Normal	Killed
G. P. ♂ 82*		62	"	Normal	?	Biopsy
G. P. ♀ 87*		61	"	"	?	"
G. P. ♀ 73		75	"	"	+	Killed
G. P. ♀ 75		141	++	+++	Normal	Died
G. P. ♀ 118		165	Normal	+	?	Biopsy, still alive
G. P. ♀ 121		145	++	++++	+	Moribund, killed
G. P. ♂ 125		107	Normal	++++	Hemorrhage	Died
G. P. ♀ 126		133	++	++++	Normal	"
G. P. ♂ 128		87	+++	++++	"	Moribund, killed
R. 7	475	30	Normal	Normal	"	Killed
R. 9	340	32	"	+	+++	"
R. ♀ 19	610	59	+++	+++	Normal	Died
R. ♂ 20	590	56	+++	++++	++	Moribund, killed
R. ♂ 21	500	56	+++	+++	++	Died

* Whole wheat substituted for bran in Diet 11.

of the muscular dystrophy. Upon microscopic examination during this stage their muscles revealed no lesions. Those animals however, which were allowed to survive for a sufficiently long period on Diet 11, developed flabbiness, muscular weakness, and a break in the growth curve, followed by rapid decline and death. At autopsy they showed very characteristic gross and microscopic lesions in the muscles.

Table V shows that five guinea pigs, killed after 31 to 75 days on the diet, had normal muscles, while six, that were allowed to survive as long as possible, showed well defined and extensive muscular dystrophy.

In rabbits the onset and severity of the muscular disorder was found to be the same for animals on the untreated Diet 11 as on Diet 13. Table V includes five rabbits, four of which had typical symptoms, gross lesions, and extensive alterations in the muscle. The fifth one, Rabbit 7, was killed after 30 days on the diet, before it had exhibited any symptoms.

The similarity in the growth curves of rabbits on Diet 11 and on Diet 13 with and without wheat germ oil, shown in Chart 5, illustrates the ineffectiveness of vitamin E administration in controlling the muscular disorder.

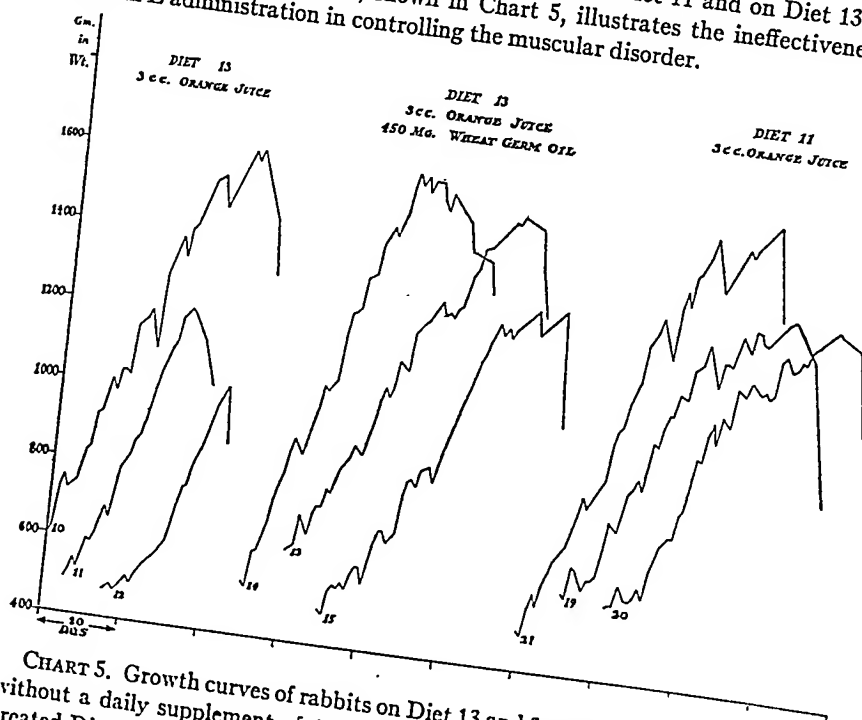


CHART 5. Growth curves of rabbits on Diet 13 and 3 cc. of orange juice, with and without a daily supplement of 450 mg. of wheat germ oil; and of rabbits on untreated Diet 11 and 3 cc. of orange juice.

It seems evident that the addition of vitamin E to Diet 13 failed to protect guinea pigs and rabbits against muscular dystrophy, and that the ethereal ferric chloride treatment of the diet plays no rôle in the production of the lesions.

The possible protective effect of green foodstuffs is now being investigated.

CONCLUSIONS

A diet is described, which leads to a progressive, highly selective, and ultimately fatal dystrophy of the voluntary muscles. Guinea

pigs and rabbits are susceptible, rats resistant. The diet used is complete in known requirements, except for vitamin E; the addition of this factor, however, does not prevent the development of the disease. The lesions are not due to inanition, infection, or scurvy, and must be referred to some still unknown factor.

It is a pleasure to acknowledge the continued interest and advice of Dr. H. T. Clarke and Dr. G. L. Foster in this investigation.

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EXPLANATION OF PLATES

PLATE 18

FIG. 1. Guinea Pig 126. 133 days on Diet 11 plus orange juice. Section of abdominal muscles, showing hyaline necrosis of fibers, with segmentation and disruptions.

FIG. 2. Rabbit 1. 14 days on Diet 13 plus orange juice. Necrotic fiber in center. Marked proliferation of muscle nuclei between still intact fibers.

FIG. 3. Rabbit 20. 56 days on Diet 11. Invasion of necrotic fiber by polymorphonuclear leucocytes.

FIG. 4. Rabbit 1. 14 days on Diet 13 plus orange juice. Regenerating fibers.

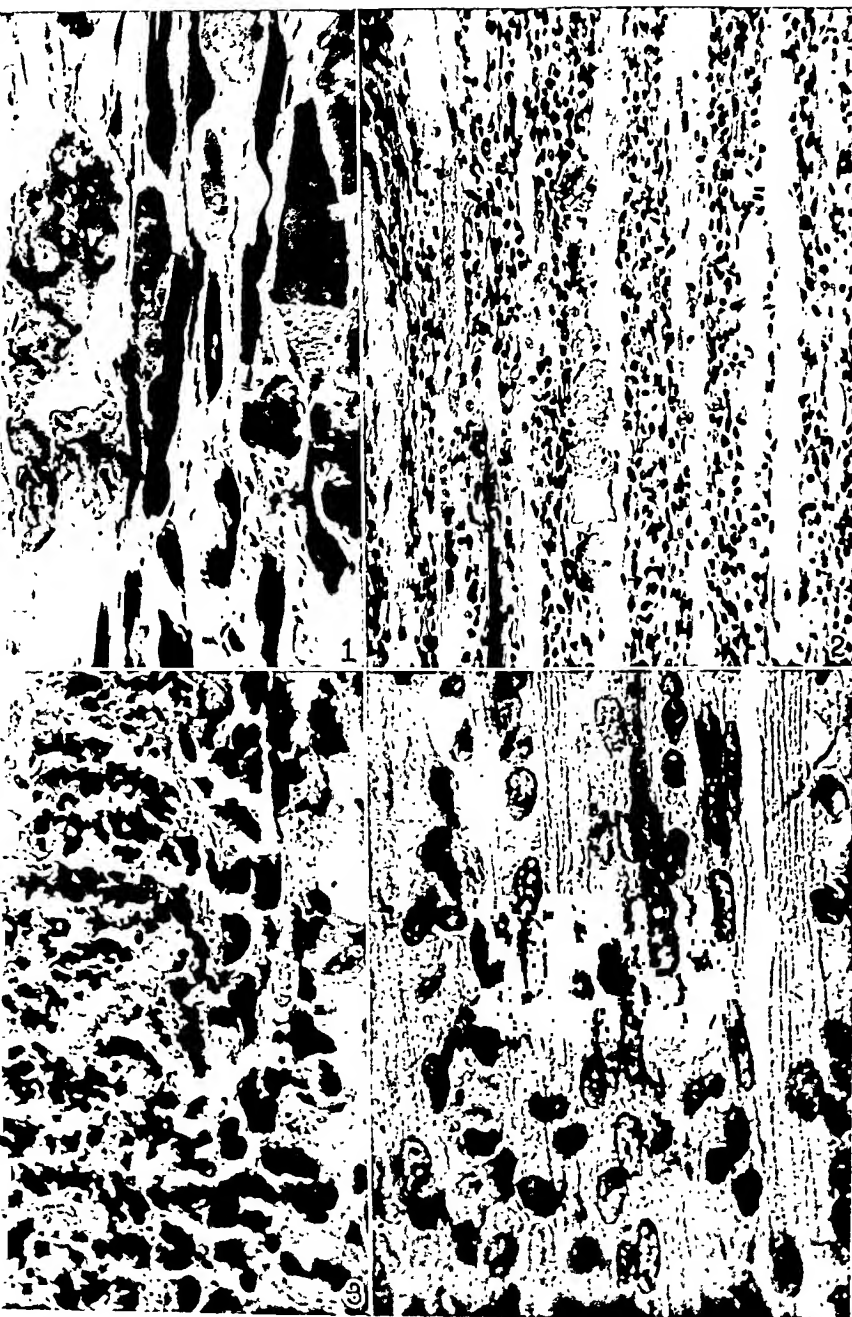
PLATE 19

FIG. 5. Guinea Pig 113. 51 days on Diet 13 plus orange juice. Extreme degeneration and loss of muscle fibers, multiplication of muscle nuclei, and formation of large multinucleate plasmatic masses.

FIG. 6. Rabbit 15. 55 days on Diet 13 plus wheat germ oil. Calcification of necrotic fibers.

FIG. 7. Guinea Pig 109. 218 days on Diet 13. Quadriceps femoris muscle, showing extreme atrophy of muscle fibers and replacement with adipose tissue.

FIG. 8. Guinea Pig 58. 122 days on Diet 13 plus 3 cc. orange juice. Section through incisor root, showing normal pulp, odontoblasts, predentine, dentine, and enamel.



(Goettch and Pappenheimer: Nutritional muscular dystrophy)



(Goettsch and Pappenheimer: Nutritional muscular dystrophy)

NERVE ENDINGS IN NUTRITIONAL MUSCULAR DYSTROPHY IN GUINEA PIGS

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PLATES 20 AND 21

(Received for publication, April 2, 1931)

In a previous article (1), dealing with nutritional muscular dystrophy in guinea pigs and rabbits, it was stated that no significant alterations were found in the central nervous system or larger peripheral nerve trunks. The question as to a possible primary degeneration of the muscular nerve endings was left for further study. The following observations indicate that the nerve endings are preserved even though the muscle fibers are profoundly altered.

Technique

The method selected after comparison with several others was devised by one of the authors (2) and should be applied as follows:¹

1. Fixation in Bouin's picro-aceto-formol—3 days or longer.
 2. 70 per cent alcohol, many changes until most of the picric acid has disappeared,—24 hours or longer.
 3. 2 per cent solution of ammonia in 80 per cent alcohol—24 hours.
 4. 2 per cent solution of ammonia in 90 per cent alcohol—24 hours.
 5. Absolute alcohol—2 hours, clear in chloroform, xylol, or cedar oil.
- The tissue is then embedded in paraffin in the usual manner. Sections are cut at 10 to 30 μ . After deparaffinizing, the slides remain in 90 per cent alcohol containing 1 per cent of ammonia for 3 hours.
6. Rapid rinsing in distilled water.
 7. 40 per cent silver nitrate for 2 to 4 hours.
 8. Rinse quickly with distilled water.

¹ We wish to thank Miss Marie Norkus for her assistance in developing and applying this technique.

9. 20 per cent acid or neutral formol is poured upon slide and allowed to remain for 5 minutes, followed by 5 per cent formol for 1 minute. Pour off without rinsing.

10. Diammoniacal silver solution which has been heated to 30–55°C. is poured *over* the slide which is held at an incline. Then more of this solution is poured *on* the slide which is placed horizontally under the microscope so that the liquid does not run off. Allow this to remain until the neurites are stained black while the section as a whole is a golden brown. The diammonical silver solution is made as follows:

To 4 cc. of 20 per cent silver nitrate add several drops of ammonium hydrate (28 per cent) to precipitate the silver. Then, with constant shaking, continue adding ammonium hydrate drop by drop until the precipitate is redissolved. Following this, add 1 drop of ammonium hydrate to every 2 cc. of the original 20 per cent solution of silver nitrate. To this add 4 cc. of distilled water.

11. Rinse in distilled water.

12. Tone with solution of gold chloride made as follows:

1 gm. gold chloride to 300 cc. distilled water. Add 50 drops of glacial acetic acid to every 5 cc. of gold chloride solution. Allow sections to stay in the toning solution for 10 to 15 minutes. The acetic acid bleaches the connective tissue.

13. Wash with distilled water.

14. Reduce the gold in 1 per cent oxalic acid for about 5 minutes, or until the sections become purple.

15. Fix in 5 per cent sodium hyposulfite for about 5 minutes.

16. Wash in running water or in several changes of water on the slide. Failure to wash well often causes stains on the sections.

17. Dehydrate; clear in carbol-xylol, then xylol, and mount in balsam.

Using this method, the nerve terminals are brought out with great clarity against the purplish muscle fibers, in which the striations are distinctly shown.

As is seen in Fig. 1, even the finest axons are impregnated, and can be followed to their terminations. They appear to end upon the surface of muscle fibers which have undergone complete hyaline necrosis. Such neurites show no obvious differences from those leading to normal muscle fibers.

Although it might seem probable that the complete disintegration and disappearance of the muscle fibers would lead to regressive changes in the nerve terminals, we have found no evidence that such is the case. On the contrary, beautifully preserved neurites, with terminal pads, may be seen amongst the columns of proliferative muscle nuclei (Figs. 2 and 3), and in the fat and connective tissue

which separates the still intact muscle fibers. It seems certain that the nerve terminals persist even after the fibers which they supply have undergone resorption and have been replaced by adipose and fibrous tissue.

It seems unnecessary in this note to review the rather contradictory literature upon the normal and pathological structure of the nerve endings in the muscles. The work upon the normal histology of motor nerve terminals has recently been summarized by Okamura (3). Tsunoda (4) has described swelling and increased tortuosity, followed by atrophy of the terminal branches and of the end-net of the axis cylinder, in experimental polyneuritis of the pigeon. We could find no similar changes in our preparations.

CONCLUSION

The nutritional muscle dystrophy of guinea pigs and rabbits is primarily a degeneration of the muscle fibers and is not associated with visible alterations of the peripheral nerves or their motor terminals.

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EXPLANATION OF PLATES

PLATE 20

FIG. 1. *a, b, c.* Guinea Pig 121. 145 days on Diet 11. Quadriceps femoris muscle. Rogers' stain. Preparation by Dr. Abner Wolf. Coarse and fine (sympathetic?) nerve fibers are seen to end upon the surface of the necrotic fibers.

PLATE 21

FIG. 2. Guinea Pig 115. 137 days on Diet 13 plus orange juice. Quadriceps femoris muscle showing loss of fibers, replacement of them by fat, multiplication of muscle nuclei, and persistence of neurites amongst the cell columns.

FIG. 3. Same muscle. Intact terminal arborizations in degenerated area. Oil immersion.



FIG. 1 *a*

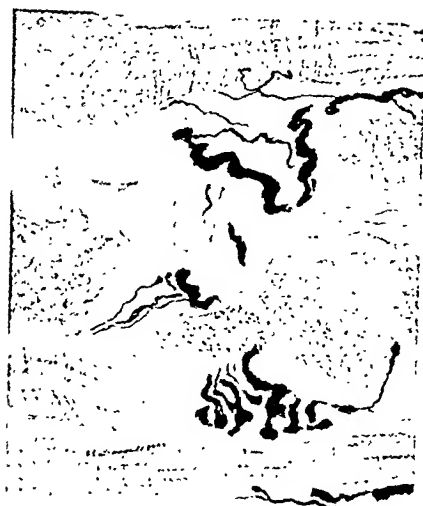


FIG. 1 *b*



FIG. 1 *c*

(Rosen *et al.* Nerve endings in muscular dystrophy)

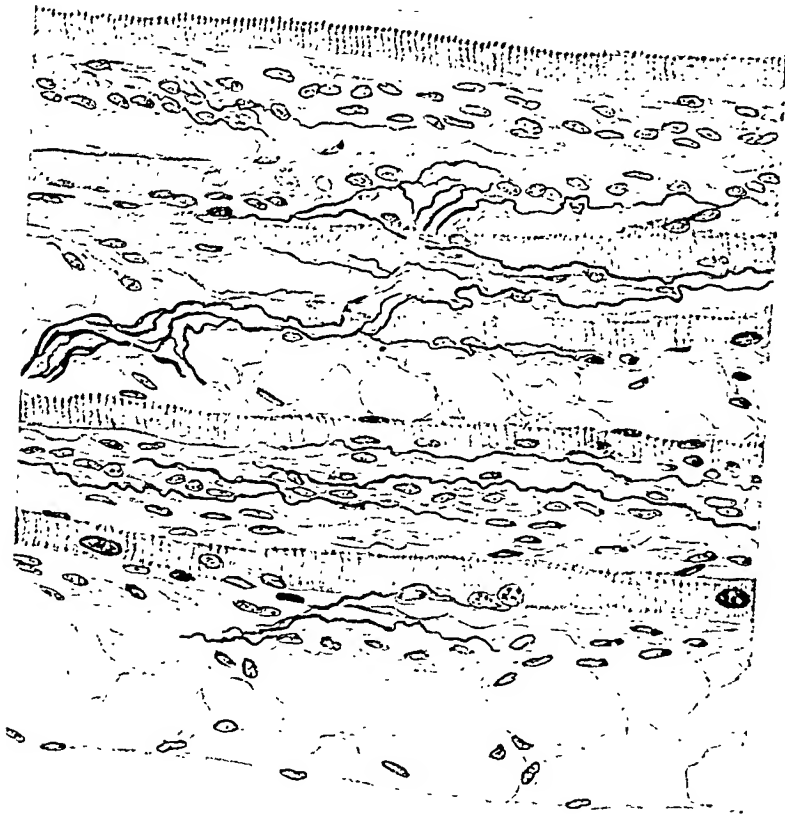


FIG. 2



FIG. 3

(Reproduced from "Nerve changes in muscular dystrophy")

REACTION OF YOUNG AND ADULT RABBITS TO PNEUMOCOCCI INJECTED INTO THE SKIN

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(Received for publication, April 10, 1931)

During recent years chance observations and experimental studies have established the fact that no inflammation or only a comparatively slight one results when irritants are introduced into the skin of very young animals.

Coca, Russell and Baughman (1) noticed that guinea pigs weighing less than 400 gm. react slightly and irregularly to the injection of diphtheria toxin into the skin. Freund (2) and Valtis (3) demonstrated that tuberculous guinea pigs less than 1 month old, do not react at all or only very slightly to tuberculin in the skin test although tuberculous guinea pigs of this age are about as sensitive to the toxic action of old tuberculin injected into the peritoneal cavity as adult tuberculous guinea pigs. Young rabbits do not react with the Arthus reaction to egg white or horse serum (4). Friedberger and Heim (5) made interesting observations on newborn infants and rabbits. They found that no inflammation or but a very slight one results when such powerful irritants as eel serum or mustard oil are injected into the skin of new-born infants or rabbits 1 or 2 days old. The extensive experiments of Adelsberger (6) showed very clearly that young infants react very slightly to turpentine and other irritants when applied to the skin. The slight reactivity of the skin of infants is significant both from immunological and epidemiological points of view. This is demonstrated by the following observations. Cook, Keith and Ermatinger (7) found that the skin of infants may not react to the injection of a relatively large amount of scarlet fever toxin in spite of the absence of scarlet fever antitoxin in the blood. Von Groer and Kassowitz (8) and Tschertkow and Belgowskaja (9) have shown that in very young infants the Schick test is negative and according to Tschertkow and Belgowskaja this test is negative even in the absence of diphtheria antitoxin in the blood. Tschertkow (10) has also observed that infants do not react with inflammation in an autolysate of typhoid vaccine is injected into the skin although such an injection usually produces inflammation in adults. Selter (11) has reported that young infants are not sensitive to intracutaneous injections of extracts of various bacteria (typhoid, dysentery, colon, *prodigiosus*).

In view of these observations systematic experiments have been undertaken to determine the reaction of very young animals to infection.

EXPERIMENTAL

To study the infection of the skin in young and adult animals pneumococcus was chosen for it had been shown by Zinsser (12),

TABLE I

Effect of Pneumococci Injected into the Skin of Adult and Young Rabbits

	No. of rab- bit	Weight	Amount of culture injected	Local inflammation	Pneumococci in blood smears	Fate of animal
		gm.	cc.			
Adult rabbits	1	2200	0.001	Typical with necrosis	Not found	Survived
	2	2500	0.001	Typical with necrosis	Found	Died in 6 days
	3	2600	0.001	Typical without ne- crosis	Not found	Survived
	4	3500	0.001	Typical with necrosis	Not found*	Died in 4 days
Rabbits 17 days old	1	140	0.001	None	Found	Died in 1 day
	2	150	0.001	Slight redness, no swelling	Found	Died in 2 days
	3	170	0.001	Slight redness, no swelling	Found	Died in 2 days
	4	175	0.001	Slight redness, no swelling	Found	Died in 2 days
	5	175	0.001	Slight redness, no swelling	Found	Died in 2 days

* Gelatinous pneumonia; pneumococci not found in the lungs.

Goodner (13) and Klinck (14), that a small amount of a culture of virulent pneumococcus injected into the skin of (adult) rabbits produces a very intense inflammation which, if certain dosage is used, heals as a rule in about 10 days. The typical inflammation was described in detail by Goodner.

In the experiments to be described observations were made on 39 young and 22 adult rabbits.

The age of the young rabbits ranged from 1 to 26 days. They were kept with the mothers. The age of the adult animals was not known. They weighed from

2000 to 3500 gm. and can be considered as fully grown rabbits. Most of the rabbits were of common laboratory stock.

The pneumococcus culture was a freshly isolated strain of Type I; it was grown on rabbit blood bouillon. The amount injected varied from 0.000016 to 0.005 cc. of the culture diluted to the volume of 0.1 cc. Previous to the injection the hair over the skin of the whole abdomen was either shaved or cut with scissors. The

TABLE II
Effect of Pneumococci Injected into the Skin of Adult and Young Rabbits

	No. of rabbit	Weight	Amount of culture injected	Local inflammation	Pneumococci in blood smears	Fate of animal
		gm.	cc.			
Adult rabbits	1	2600	0.0005	Typical without necrosis	Not found	Survived
	2	2600	0.0005	Typical with necrosis	Not found	Survived
	3	3400	0.0005	Typical without necrosis	Not found	Survived
	4	3600	0.0005	Typical without necrosis	Not found*	Died in 15 days
Rabbits 7 days old	1	70	0.005	None	Found	Died in 1 day
	2	70	0.005	None	Found	Died in 1 day
	3	75	0.005	None	Found	Died in 2 days
	4	80	0.005	None	Found	Died in 1 day
	5	80	0.005	None	Found	Died in 1 day
	6	80	0.005	None	Found	Died in 1 day
	7	80	0.005	None	Found	Died in 1 day
	8	85	0.005	None	Found	Died in 1 day
	9	85	0.005	None	Found	Died in 1 day
	10	90	0.005	None	Found	Died in 1 day
	11	100	0.005	None	Found	Died in 1 day

* Pneumonia; pneumococci not found in the lungs.

animals were observed for 2 weeks, blood smears were examined for pneumococcus daily (with a few exceptions). The results are tabulated in Tables I to VI.

In each experiment, represented in the tables, one group of young rabbits and one group of adults were injected with the same suspension. In the first experiment 0.001 cc. of the culture was injected in both groups. All four adult rabbits had typical local inflammation; one rabbit died in 6 days with bacteriemia, one other rabbit died in 4 days from pneumonia without bacteriemia. The death of the latter rabbit was caused probably not by the injection of pneumococci. Both of these rabbits had typical local inflammation. None of the young rabbits 17 days

old, reacted locally in the way that is characteristic for adult rabbits. The skin was very slightly red only, and no swelling was noticed. Of the young rabbits one died in 1 day and the other four rabbits in 2 days after the infection. The blood of all these contained a large number of extracellular and relatively few intracellular pneumococci (Table I).

In the second experiment four adult and eleven young rabbits, 7 days old, were infected with 0.005 cc. of the culture. All adult rabbits had typical local inflammation; one of them died on the 15th day after infection from pneumonia. None

TABLE III
Effect of Pneumococci Injected into the Skin of Adult and Young Rabbits.

	No. of rabbit	Weight	Amount of culture injected	Local inflammation	Pneumococci in blood smears	Fate of animal
		gm.	cc.			
Adult rabbits	1	2600	0.005	Typical without necrosis	Found	Died in 3 days
	2	2900	0.005	Typical without necrosis	Not found	Survived
	3	2900	0.005	Typical with necrosis	Not found	Survived
	4	3100	0.005	Typical with necrosis	Not found	Survived
Rabbits 23 days old	1	200	0.005	None	Found	Died in 2 days
	2	210	0.005	Slight redness, no swelling	Found	Died in 2 days
	3	210	0.005	Slight redness, no swelling	Found	Died in 3 days
	4	240	0.005	Slight redness, no swelling	Found	Died in 3 days
	5	245	0.005	Slight redness, swelling 2 mm. thick	Found	Died in 3 days
	6	255	0.005	None	Found	Died in 3 days
	7	255	0.005	None	Found	Died in 3 days
	8	258	0.005	Slight redness, no swelling	Found	Died in 3 days

of the blood smears of these rabbits showed pneumococci. Of the eleven young rabbits, ten died on the day following the injection and one died 2 days after the injection. All of the young rabbits had pneumococci in their blood. Local inflammation was not noticed (Table II).

In the third experiment four adult and eight young rabbits were infected. The young rabbits were 23 days old. Both groups were infected with 0.005 cc. of the culture. One of the adult rabbits (No. 1) died 3 days after the infection. In the blood smears taken on the 1st, 2nd and 3rd day after the infection there were

many pneumococci both intra- and extracellular. It should be emphasized that the local inflammation was typical. So that in this rabbit the changes that led to death did not modify the development of the local inflammation. Of the young rabbits two died in 2 days and six died in 3 days. The blood smears of all the rabbits contained pneumococci, mostly extracellular. Three of these rabbits showed no local reactions at all; the skin of four rabbits was slightly red without being swollen, and the skin of one young rabbit was red and very slightly thickened (Table III).

In the fourth experiment four adult rabbits were infected with 0.001 cc. and the five young rabbits with 0.000016 cc. of the culture. All the adult rabbits had typical local inflammation and survived the infection without septicemia. None

TABLE IV
Effect of Pneumococci Injected into the Skin of Adult and Young Rabbits

	No. of rabbit	Weight	Amount of culture injected	Local inflammation	Pneumococci in blood smears	Fate of animal
		gm.	cc.			
Adult rabbits	1	2200	0.001	Typical with necrosis	Not found	Survived
	2	2900	0.001			
	3	2900	0.001			
	4	3500	0.001	Typical without necrosis	Not found	Survived
Rabbits 8 days old	1	70	0.000016	Typical with necrosis	Not found	Survived
	2	100	0.000016	None		
	3	110	0.000016	None		
	4	110	0.000016	None	Found	Died in 3 days
	5	110	0.000016	None		
					Found	Died in 4 days
					Found	Died in 4 days
					Found	Died in 4 days
					Found	Died in 5 days

of the young rabbits showed any local inflammation. All died from 3 to 5 days after the infection with septicemia (Table IV).

In the fifth experiment six adult and ten young rabbits were injected. The young rabbits were 26 days old. In this experiment the amount of culture injected was varied in both groups of rabbits.

Two adult rabbits were infected with 0.005 cc., two with 0.0005 cc. and two with 0.00025 cc. of the culture. All these rabbits developed typical local inflammation; pneumococcus was found in the blood of only one, namely in the rabbit that died 3 days after the infection. Of the young rabbits six were infected with 0.0005 cc. and four with 0.00012 cc. of the culture. In some of these rabbits there was found only slight redness and no swelling at all, and in four rabbits there was a definite edema noticed. Rabbit 4 showed the most conspicuous reaction: slight redness and swelling over an area 30 x 20 mm., elevated about 6 mm. All of these rabbits died with septicemia from 3 to 6 days after infection (Table V).

Summary of the Five Experiments

Adult Rabbits.—Six rabbits were injected intracutaneously with 0.005 cc., eight with 0.001 cc., six with 0.0005 and two with 0.00025 cc.

TABLE V

Effect of Pneumococci Injected into the Skin of Adult and Young Rabbits

	No. of rabbit	Weight	Amount of culture injected	Local inflammation	Pneumococci in blood smears	Fate of animal
		gm.	cc.			
Adult rabbits	1	2200	0.005	Typical with necrosis	Not found	Survived
	2	2500	0.005	Typical with necrosis	Not found	Survived
	3	2600	0.0005	Typical with necrosis	Not found	Survived
	4	2900	0.0005	Typical without necrosis	Found	Died in 3 days
	5	2950	0.00025	Typical without necrosis	Not found	Survived
	6	3000	0.00025	Typical without necrosis	Not found	Survived
Rabbits 26 days old	1	300	0.0005	Slight redness, no swelling	Found	Died in 3 days
	2	310	0.0005	Slight redness, no swelling	Found	Died in 3 days
	3	315	0.0005	Slight redness, no swelling	Found	Died in 3 days
	4	315	0.0005	Slight redness, swelling 30 x 20 mm. raised 6 mm.	Found	Died in 3 days
	5	325	0.0005	Redness, no swelling	Not found	Died in 6 days
	6	325	0.0005	Slight redness, no swelling	Found	Died in 7 days
	7	330	0.00012	Redness, no swelling	Found	Died in 3 days
	8	330	0.00012	Redness, swelling 12 x 12 mm., raised 3 mm.	Found	Died in 3 days
	9	340	0.00012	Redness, swelling 15 x 15 mm., raised 2 mm.	Found	Died in 3 days
	10	340	0.00012	Redness, swelling 40 x 40 mm., raised 2 mm.	Found	Died in 6 days

of a pneumococcus culture. All of them developed a very extensive local inflammation; three of the rabbits died with septicemia and two without septicemia.

Young Rabbits, from 1 to 26 Days Old.—Nineteen young rabbits were injected intracutaneously with 0.005 cc., five with 0.001 cc., six with 0.0005 cc., four with 0.00012 cc. and five with 0.000016 cc. of a pneumococcus culture. None of these rabbits developed the typical local inflammation. Redness with slight swelling was found only in five rabbits, redness without swelling was noticed in fourteen and no

TABLE VI

Effect of Pneumococci Injected into the Skin of Adult and Young Rabbits
Adult Rabbits

No. of rabbits	Dose	Local inflammation	Survived	Died with septicemia	Died without septicemia
	cc.				
6	0.005	Typical	5	1	0
8	0.001	Typical	6	1	1
6	0.0005	Typical	4	1	1
2	0.00025	Typical	2	0	0

Young Rabbits

No. of rabbits	Dose	Local inflammation			Survived	Died with septicemia	Died without septicemia
		Absent	Redness, no swelling	Redness and swelling			
	cc.						
19	0.005	14	4	1	0	19	0
5	0.001	1	4	0	0	5	0
6	0.0005	0	5	1	0	5	1*
4	0.00012	0	1	3	0	4	0
5	0.000016	5	0	0	0	5	0

* 26 days old.

evidence of inflammation was seen in twenty rabbits. All young rabbits died with bacteriemia (Table VI).

DISCUSSION

Goodner and others have demonstrated that the injection of virulent pneumococci into the skin of adult rabbits produces a very extensive inflammation which in some of the animals heals in about 10 days. In others the pneumococci penetrate into the blood stream and cause

death. With the strain and amounts used in the experiments reported in the present paper about one of seven adult rabbits died with septicemia. We have found that young rabbits, from 1 to 26 days old, react very differently to the intracutaneous injection of pneumococci. The inflammation at the site of injection either fails to develop or it is very slight and in all animals without exception the pneumococci penetrate into the blood and kill the rabbits. It was noticed that the pneumococci found in the blood of adult rabbits are mainly in the leucocytes, whereas in the blood of young rabbits they are mainly extracellular.

The observation that young rabbits fail to develop extensive inflammation might be explained by their severe septicemia and by the fact that they live only for a short time after they have been infected. However in those few adult rabbits that died within a few days after infection the septicemia was associated with the usual inflammation at the site of the injection of pneumococci. This observation is in accord with the experiments of Goodner.

In our first experiments an amount of culture was injected both into the young and adult rabbits which was suited to produce extensive inflammation without killing the majority of adult rabbits. In subsequent experiments the amount was varied so that the doses were proportional to the body weights of young and old. Even when this was done, a very small amount of culture, relatively speaking, as little as 0.000016 cc., killed the young rabbits, with septicemia. Since the mortality of young rabbits due to various intercurrent diseases is high (and very frequently the cause of death is not found at autopsy) it is possible that some of the young rabbits would have died during the course of the experiments without having been infected with pneumococci.

The failure of young rabbits to develop extensive inflammation at the site of injection was expected on the basis of observations on the slight reactivity of the skin of young animals. The possible relation of the presence and extent of inflammation to the fate of the animals is of interest. The adult animals develop extensive inflammation and the large majority of them survive the infection without developing a septicemia. The young rabbits do not have inflammation at the site

of the injection, or the inflammation is very slight, and all die with large numbers of pneumococci in the blood stream. It is very probable that the penetration of the bacteria from the skin into the blood stream and the fate of animals is determined by the inflammation at the site of injection of pneumococci. This assumption is supported by experiments which have shown that by means of artificially induced inflammation the spread of bacteria can be checked and animals can be saved from otherwise fatal infection. Opie (15) in a study on the relation of inflammation to immunity found that if, before the injection of streptococci into the peritoneal cavity of rabbits, peritonitis is induced by injection of aleuronat the animals are protected from otherwise fatal bacteriemia.

In addition to the difference in the capacity to develop inflammation young and adult rabbits may differ in regard to the readiness with which non-irritating substances penetrate into the blood from the skin. This may be significant in the reaction of rabbits to pneumococci injected into the skin.

CONCLUSIONS

1. Young and adult rabbits react differently to intracutaneous injection of virulent pneumococci. In adult rabbits a very extensive inflammation develops at the site of infection and bacteriemia and death occur only in a relatively few rabbits. Young rabbits fail to develop extensive inflammation and die with bacteriemia.
2. It is probable that the fate of the animals is influenced by the capacity to develop inflammation at the site of injection of pneumococci.

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TYPHUS FEVER

I. COMPARATIVE STUDY OF EUROPEAN AND AMERICAN TYPHUS IN LABORATORY ANIMALS

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(Received for publication, April 6, 1931)

American strains of typhus (Mooser (1), Maxcy (2)) differ from European strains (Nicolle (3), Wolbach (4)) chiefly in the fact that the former produce a severe acute inflammation of the scrotal sac in laboratory animals.

In a previous paper (5), attention was called to the occurrence of a periodic scrotal sac exudate in guinea pigs inoculated with the Wolbach strain of European typhus and to the presence of *Rickettsiae*-filled cells in this exudate. It therefore seemed probable that the differences between European and American typhus are unimportant and that the two diseases are essentially identical. More detailed clinical, pathological and morphological studies have since been made on four strains of typhus—two of European origin and two of American origin. The object of this paper is to record certain interesting features which were brought out in this comparative study.

The origin of these four strains¹ was as follows:

European Strain 1 (Wolbach). Established in guinea pigs in Warsaw, Poland (1919). Maintained in this laboratory up to the present time.

European Strain 2 (Breinl). Established in guinea pigs in Prague (1928). Received in this laboratory in Oct., 1929, and maintained until Sept., 1930.

Mexican Strain (Mooser). Established in guinea pigs in Mexico (1928). Received in this laboratory in Oct., 1928, and maintained until Jan., 1930.

North Carolina (U. S. A.) Strain (Maxcy). Established in guinea pigs in Wilmington, N. C. (1928). Received in this laboratory in Sept., 1929, and maintained up to the present time.

¹ I am indebted to the originators of the various strains for the privilege of making these studies.

The differences between the four strains, and the more important points of similarity are brought out concisely in Table I.

In all four strains the characteristic brain lesions were demonstrated. In the two American strains, however, brain lesions were practically absent in male guinea pigs which had been inoculated intraperitoneally and in which the characteristic acute inflammation of the scrotal sac had occurred. In female guinea pigs (in which no marked local reaction takes place) or in male guinea pigs inoculated subcutaneously, brain lesions were usually found fairly easily, although they were not as numerous as in the European strains. In six male guinea pigs inoculated subcutaneously with a minute amount of infectious material (scrotal sac exudate, Maxcy strain) brain lesions were fully as numerous as in the European strains. In order to obtain numerous brain lesions in the American strains it would seem that we must prevent the occurrence of a severe local reaction.

In all four strains, *Rickettsiae*-laden cells were found in the scrotal sac exudate. In the two American strains (which were alike in all respects as far as could be learned) there was always a copious exudate in the scrotal sac, and *Rickettsiae*-filled cells were found easily in every case. In the Wolbach European strain, the exudate was usually slight in amount and a careful search was necessary in order to find the specific organisms. During a period of intensification of the scrotal reaction, however, infected cells were found practically as easily as in the American strains. In the Breinl strain, the exudate in the scrotal sac was always scanty, and a large number of attempts were made before the characteristic picture was found. When found, however, the appearance of the intracellular organisms did not differ from that seen in the other strains.

These two facts—the presence of characteristic brain lesions in all strains and the presence of *Rickettsiae*-filled cells in the scrotal sac in all strains, leave no doubt concerning the essential similarity of the four strains studied. As far as can be learned, all strains established from endemic sources have shown this marked local reaction in the scrotal sac of the guinea pig, while all strains established from epidemics of high mortality have, when first established, shown a mild inconspicuous scrotal reaction and more numerous brain lesions. It therefore seems justifiable to conclude that variations in the intensity of the

TABLE I
Comparative Study of Four Strains of Typhus

Strain.....	European No. 2 (Breinl)	European No. 1 (Wolbach)	Mexican (Mooser) Wilmington (Maxcy)
Incubation period	5-7 days up to 21 days with very small dose	7-10 days, up to 21 days. Reduced to 5 days with scrotal sac exudate	2-19 days depending on dose
Swelling of scrotum after intraperitoneal inoculation	Never observed definitely	Rare. Periodic. Transient (6-24 hrs.). May be induced by passage through rats	Practically constant
Scrotal sac exudate after intraperitoneal inoculation	Frequent at end of incubation period. Never copious	Practically always at end of incubation period. Often copious	Always copious
Scrotal sac adhesions	Temporary, rare. Permanent, never	Temporary, often. Permanent, rare	Usually permanent
<i>Rickettsiae</i> -filled cells in scrotal sac exudate	Found only in comparatively small numbers	Few to many, depending on amount of exudate present	Numerous in every case
Exudate on spleen	Frequently	In about 60% of cases	Occasionally
<i>Rickettsiae</i> -filled cells in exudate on spleen	Found in two preparations after long search	Rare. One or two cells found after long search	As in other strains.
Brain lesions	Constant and usually numerous	Constant and usually numerous	Frequent in female pigs. In male pigs, only after subcutaneous inoculation

scrotal reactions are correlated with strain variations in virulence similar to those shown by various members of the streptococcus group.

The behavior of the Wolbach strain of European typhus during its 12 year residence in the guinea pig is worthy of special comment. During the first 2 years after this strain was established, a large number of guinea pigs were autopsied and their scrotal sacs carefully examined. Involvement of the testes was confined to an occasional small area of superficial hemorrhage and no obvious exudate was ever found. During the next 7 years autopsies were made from time to time but a mild scrotal sac exudate might have escaped notice. No obvious involvement of the scrotal sac was noted during this period. 3 years ago, when the scrotal reaction was first noticed, as it was mild, transient, of rare occurrence and not visible externally. During the past 3 years the scrotal reaction has become more pronounced and more frequent (although it still occurs only periodically). Recently the reaction has been entirely comparable to that seen in American typhus in four successive transfers, but it practically disappeared again in the fifth transfer. At present it has been practically absent for six generations (about 2 months). This European strain has therefore been definitely altered by its long residence in guinea pigs and now occupies a position intermediate between that of a recently isolated European strain and the American strains (see Table I).

The scrotal reaction in the Wolbach strain was intensified during quiescent periods, in the following way: A rat was inoculated intraperitoneally with 1 cc. of brain emulsion from a typhus-infected pig (without scrotal reaction). 14 days later the spleen and testicles of the rat were ground in a mortar and two guinea pigs were inoculated with this material. This procedure was carried out four times and in each case at least one of the guinea pigs inoculated from the rat showed a marked scrotal reaction with complete fixation of the testes in the scrotal sac. Whether this phenomenon depends on increased dosage or a true change in virulence of the organism is not clear but the following observations indicate that the latter is more probable.

On numerous occasions when a copious exudate with many *Rickettsiae* was found in the scrotal sac in the Wolbach European strain, large amounts of this exudate were injected intraperitoneally into normal guinea pigs. The percentage of strongly positive scrotal

reactions obtained by this method was no greater than in the routine transfers with heart's blood. This seems to indicate that the periodic variation in the scrotal reaction in this strain does not depend on increased dosage but rather upon some unknown factor.

It has been interesting to study the incubation periods of the disease in guinea pigs in the various strains after inoculation by various routes. In the Wolbach strain, routine inoculations of 4 cc. of blood on the 3rd or 4th day of fever resulted quite regularly in an incubation period of 9 or 10 days (rarely and somewhat periodically shortening to 8 days or lengthening to 12 days). In those cases which showed a marked scrotal reaction after blood inoculation, the incubation period was usually 6 or 7 days. By inoculating with scrotal sac exudate, the incubation period was quite regularly 5 or 6 days irrespective of whether a scrotal reaction occurred. In other words, inoculation with a heavy dose of *Rickettsiae* (a generous quantity of scrotal sac exudate) decreased the incubation period markedly but did not appear to increase the intensity of the scrotal reaction.

In the Breinl strain, when transfers were made with blood (4 cc. intraperitoneally) the incubation period ranged from 5 to 7 days. On two occasions when scrotal sac exudate containing visible *Rickettsiae* in moderate numbers was used for inoculation, the incubation period was 5 days and the type of reaction did not differ from that regularly seen after intraperitoneal inoculation with blood.

In the Mexican or North Carolina strains, the incubation period when blood was used for transfer showed great variations (5 to 18 days) and a negative result was obtained much more often than with the Wolbach strain. This was undoubtedly due to the fact that the local reaction in the scrotal sac often results in partial or complete sterilization of the blood stream. When scrotal sac exudate was used for the transfer, the incubation period varied from 24 hours to 16 days, and the period could be fairly accurately predicted by making smears of the exudate and noting the number of *Rickettsia*-filled cells present. When infected cells were present in every low power field, the incubation was never less than 4 days. When a long search was necessary the incubation period was never less than 7 days and when *Rickettsiae* were not found at all, it was only on one occasion less than 10 days.

The evidence indicates that the incubation period is dependent on the number of *Rickettsiae* injected rather than on virulence factors.

SUMMARY

Study of two strains of epidemic (European) typhus and two strains of endemic (American) typhus in laboratory animals has shown their essential identity.

The characteristic typhus brain lesions can be shown to occur in all four strains after subcutaneous inoculation and *Rickettsiae*-filled cells can be found in the scrotal sac in all four strains after intraperitoneal inoculations.

A strain of European typhus, which for 8 years has yielded no obvious scrotal reaction in guinea pigs, afterwards gave rise to a periodic scrotal reaction of variable severity. At present it occupies an intermediate position in this respect between the recently isolated European strain and the American strains.

Variations in the scrotal reaction in guinea pigs appear to be correlated with strain variations in virulence in the human host.

The incubation period in guinea pigs after intraperitoneal inoculation depends largely on the number of *Rickettsiae* injected.

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TYPHUS FEVER

II. CYTOLOGICAL STUDIES OF THE SCROTAL SAC EXUDATE IN TYPHUS-INFECTED GUINEA PIGS

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PLATES 22 AND 23

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INTRODUCTION

The scrotal reaction in guinea pigs reacting to Mexican typhus was first described by Neill (1) in 1917. Mooser (2) in 1918 made the important observation that the scrotal sac exudate in animals showing this reaction contained cells greatly distended with minute diplobacilli. These organisms are entirely similar to those seen in the epithelial cells lining the gut of the typhus-infected louse and to those seen in the endothelial cells of specific typhus lesions in man and laboratory animals, (Wolbach, Todd and Palfrey (3)). The etiological relationship of this organism (*Rickettsia prowazeki*) to typhus must be regarded as established.

Maxcy (4) also found *Rickettsiae*-laden cells in the scrotal sac exudate in guinea pigs infected with endemic typhus in southeastern United States. The author (5) found an entirely similar picture in the Wolbach strain of European typhus, but the inflammation of the scrotal sac was usually milder in this strain and occurred only periodically. Previous to these observations, *Rickettsiae* could be demonstrated in mammalian tissues only in small numbers, and the reaction to the organism had always been described as a proliferative response on the part of the vascular endothelium.

Cytological study of the scrotal sac exudate has brought out several interesting points which it is the object of this paper to record. These observations have been made on the Maxcy strain of typhus originating in North Carolina, instead of on the European strain because one

can more easily and constantly get material containing numerous infected cells from this strain; but unquestionably the observations could, with patience, have been made on the Wolbach European strain by taking advantage of the periodically intensified scrotal reaction. The main objects of this study were (1) to determine the origin and nature of the various types of cell present in the exudate and (2) to determine to what extent the various types of cell take up *Rickettsiae* and to what extent the organism multiplies in each type of cell. Incidentally it was hoped that such studies might help to solve the question of whether or not the peritoneal lining cells give origin to any important number of "peritoneal phagocytes."

OBSERVATIONS

In the gross the exudate is not remarkable in appearance. In the early stages it is a glairy greyish white material, in which small white flakes (up to 1 mm. in greatest dimension) are frequently found. Later (after 24 to 48 hours) the exudate becomes firmer and drier and loosely glues together the visceral and parietal layers of tunica. At this stage it is easily peeled off in large thin sheets. By grasping it with the forceps at any point it is often possible to denude the entire surface of the testicle and cremasteric muscle. When sheets of this exudate are floated in Ringer's fluid they have a distinctly membranous appearance and when manipulated with dissecting needles, planes of cleavage appear which are always parallel to the flat surface. It is thus possible to separate the material into thin sheets only two or three cells deep.

Later the exudate either becomes partially replaced by permanent connective tissue or disappears by resolution, allowing a restoration of the serosa to normal. The latter process is the rule in the Wolbach strain of European typhus where the inflammation is relatively mild, and the exception in the American strains.

Microscopic study of the exudate shows inflammatory cells of all types and relatively little fibrin. In the early stages considerable serum is present. Table I shows roughly the composition of the exudate at various stages. The figures in this table were obtained in each case by counting 1000 cells in Giemsa-stained smear preparations. The term "large mononuclear cells" is used in a descriptive sense only

and includes macrophages of whatever origin, desquamated serosal cells and perhaps a small number of young fibroblasts and true vascular endothelial cells. The serosal cells can often be identified in Giemsa-stained smears with a fair degree of certainty. In the first place they tend to occur in small clusters accurately fitted together. Secondly, the serosal cells have rather pale nuclei and in well fixed smears, several definite blue staining nucleoli are almost invariably visible, while the nucleoli of the macrophages are not seen.

In supravital preparations more accurate identification of these cells is possible. Of the macrophages about 25 per cent take up dye in the manner characteristic of the monocyte, while the remainder are

TABLE I

	Polymor- phonuclears	Eosino- philes	Lympho- cytes and plasma cells	Large mononuclear cells*
	per cent	per cent	per cent	per cent
First few hrs.	16	1.5	2.5	80
24 hrs. (approximately)	56	4.2	2.1	38
48 " "	41	3.2	6	50
4 days	10	1.2	4	85
6 "	1.2	0.2	7.5	91

* Term used in a purely descriptive sense.

probably largely of local origin (histiocytes, clasmatoocytes). The dye-storing cells constitute about 35 per cent of the "large mononuclear cells" of the exudate. The remaining 65 per cent do not store dye and are probably largely serosal cells. It is impossible to recognize young fibroblasts and true endothelial cells (originating in capillary sprouts) among these non-phagocytic cells but these types of cell must be present in very small numbers if at all in the early stages of the process, when the exudate is not adherent.

In smears made during the first 48 to 72 hours, *Rickettsia*-filled cells are practically always found, and during the first few hours of the reaction such cells are frequently present in every low power field.

Heavily infected cells are best located under low power magnification and stand out as swollen cells with dark purple cytoplasm. Occasionally the cytoplasm is so

packed with organisms that the nucleus is entirely obscured and the cells appear at first glance like masses of homogeneous darkly stained foreign material. In some of these heavily infected cells it is impossible to resolve the individual organisms except along the edge.

In other cells the nucleus appears small and dark, obviously compressed by the growth of organisms in the cytoplasm. With the oil immersion, many cells are also found which contain smaller masses of organisms. Some show one-half or one-third of the cytoplasm packed with organisms, while the uninfected portion of the cytoplasm remains clear. Even when only 15 to 20 organisms are present in a single cell, they are almost invariably in the form of a closely packed cluster, suggesting that the motility of the cytoplasm is not sufficient to disseminate them.

Unfortunately it was not possible to recognize the *Rickettsiae*-filled cells in supravital preparations. By the use of graphite ink, however, it was possible to obtain smears in which the *Rickettsiae* were plainly visible in the cytoplasm of the cells and in which the phagocytic cells could be easily recognized.

The two methods used were (1) to mix the exudate with the ink particles suspended in saline and make smears after 15 to 45 minutes, and (2) to inject the ink suspension intraperitoneally at the onset of the scrotal reaction. The latter method resulted in more satisfactory preparations. 3 drops of ink in 5 cc. of saline were injected. 24 hours after injection 50 to 80 per cent of the polymorphonuclears and 65 to 90 per cent of the macrophages (as determined by supravital preparations) were found to contain definite ink particles, and the greater number of these cells were well filled. This was used as a criterion for the success of the contact between the cells of the exudate and the ink particles.

In smears of this type, 400 *Rickettsiae*-infected cells were counted, 108 of these being completely filled with *Rickettsiae*, 134 partially filled and 158 containing from 6 to 100 individual organisms. Of these 400 infected cells, counted in smears representing four different experiments, only two contained particles of graphite and in each of these two instances it was obvious that the cells had been invaded by (or possibly were simply in contact with) polymorphonuclears and that the graphite particles were originally in the cytoplasm of the polymorphonuclears and not in the *Rickettsiae*-infected cells.

On the other hand, 2000 mononuclear cells containing ink particles were carefully inspected and no *Rickettsiae* were found in any of them. Later several hours were spent in examining these phagocytic cells without enumerating them, and no *Rickettsiae* were ever found in cells which had ingested ink particles.

No organisms were seen in the polymorphonuclears but in other smears as many as ten organisms have been counted in the cytoplasm of some of these cells. This observation had been made only very rarely, however. When the organisms are seen in polymorphonuclears, they are scattered uniformly through the cytoplasm,

as though they had been picked up one at a time. It is probable that they are rapidly destroyed.

These experiments showed in a clear-cut manner that the cells in the scrotal sac which had become infected with *Rickettsiae* and which we are forced to regard as entirely of serosal origin (see observation made on sections below) do not take up graphite ink particles and do not stain supravitaly and that the monocytes and clasmatoocytes (histiocytes) which take up graphite ink, do not take up or become invaded by *Rickettsiae* in demonstrable numbers (see Fig. 1). This latter observation agrees quite well with our repeated failure to find organisms within the circulating blood monocytes, and we believe that *Rickettsiae* gain entrance to the blood stream largely in a naked condition by way of lymphatics.

Sections through the scrotum, scrotal sac exudate and testes, stained in such a way as to bring out the *Rickettsiae* clearly, have furnished valuable material for study in connection with the above experiments.

The infected serosal cells are quite loosely attached and great care is necessary to get preparations in which they are intact. The following method has been most satisfactory. The entire scrotum, testes and rectum were removed *en bloc* and placed in Regaud's fluid (20 cc. of full strength formaldehyde solution, 1 gm. of sodium sulfate and 100 cc. of 2.5 per cent potassium dichromate solution). Equally good results were obtained with neutralized and unneutralized formaldehyde. After a few hours the scrotal sacs were gently opened from above downward to allow better access of the fixative to the visceral and parietal tunica. After 48 hours fixation, followed by gentle washing for 24 hours, suitable blocks were cut with a sharp razor, taking care not to disturb the surface exudate. The blocks were dehydrated, cleared in cedar oil and embedded in paraffin in the usual way. Sections were cut as thin as possible and stained overnight in Giemsa solution. Exposure to sunlight is the most satisfactory method of differentiation, since colophonium decolorizes many of the organisms.

In sections made in this way, the topography of the inflammatory reaction may be studied, and more information can be obtained than in a study of smears. The intracellular *Rickettsiae* stain purple and are shown fully as well as in the best smear preparations. They are stained as deeply and sharply as ordinary bacteria. No intermediate stages between definite *Rickettsiae* and granules of indefinite nature are seen, and it seems safe to assume that practically all of the *Rickettsiae* present in any given section are clearly visible.

The outstanding features of such preparations (Fig. 2) are the presence of enormous numbers of *Rickettsiae* in the cytoplasm of the serosal cells, and the extreme difficulty of finding the organism in the underlying tissue or in the cells of the overlying exudate. Organisms are never seen in an extracellular position. In many preparations, more than half of the serosal cells are completely or partially filled with *Rickettsiae*, and as many as twenty-five adjacent cells have been seen, all of which were heavily infected. Occasionally a desquamated *Rickettsiae*-containing cell is seen in the exudate near the serosa, but the cells which lie further out in the exudate (polymorphonuclears, eosinophiles, lymphocytes, macrophages, desquamated serosal cells and connective tissue cells, if any) appear uniformly *Rickettsia*-free.

There is often marked proliferation of the serosal cells, sometimes resulting in concentric whorls and sometimes simply forming a thick layer of heaped up cells, 15 to 30 cells deep. These heaped up cells tend to elongate and resemble fibroblasts, but as has been said, *Rickettsiae* are present almost exclusively in those cells which are in contact with the subserous collagen layer, or, in other words, these cells which occupy the position of the original peritoneal lining cells. Whether this is because the proliferated serosal cells have acquired immunity from their infected antecedents, or because physical or chemical changes associated with their altered position make them unsuitable for the growth of the organisms, would seem to be an important question which we have not been able to answer. Frequently when the original or "basal" layer of serosal cells has become obscured by the reaction, it can be located under low power as a layer of cells filled with purple *Rickettsiae*, just outside of the dense subserosal collagen. Figs. 3 and 4 show a localized area of serosal cell proliferation with *Rickettsiae* present only in the "basal" layer.

The proliferated serosal cells at this stage are embedded in a fibrin clot. Lateral, typical collagen appears around these cells and the transition between fibrin and collagen is difficult to follow. Dissolution of the fibrin and replacement of it by advancing granulation tissue is not seen.

The fibroblasts beneath the serosa and elsewhere likewise contain no *Rickettsiae*, and no organisms are found in fat cells, epidermis, smooth

muscle cells, striated muscle fibers, sebaceous or sweat gland cells or in the interstitial or epithelial cells of the testis and epididymis.

The reaction in the deeper tissues is primarily that characteristic of typhus in the human, namely a marked proliferation of capillary and lymphatic endothelium with occasional thrombus formation, and a marked perivascular accumulation of inflammatory cells, largely macrophages and cells of the lymphocytic series. The fibres of the cremasteric muscle undergo considerable necrosis and large numbers of polymorphonuclears accumulate about them. Frequently, also, there is degeneration of the parenchymal cells of the testis to a depth of 2 to 3 mm. with a definite purulent reaction. From the practically complete absence of *Rickettsiae* in these regions, we assume that the necrosis is caused by fairly strong toxins diffusing from the heavily infected serosal cells. The absence of gross necrosis in human tissues is probably due to the fact that only relatively small numbers of *Rickettsiae* are present in any given focus of infection.

After long search it is possible to find a few definite *Rickettsiae* in the true endothelial cells lining the capillaries and lymphatic vessels but organisms have never been found in the abundant cytoplasm of the perivascular macrophages or of the macrophages which collect in the loose areolar tissue beneath the serosa.

COMMENT

These observations lead us to conclude that in guinea pigs inoculated intraperitoneally infection with *Rickettsia prowazeki* is limited to the serosal cells *in situ* and the true capillary and lymphatic endothelial cells *in situ*. Their luxuriant growth in the serosal cells exactly parallels their behavior in the epithelial cells which line the gut of the louse. The very infrequent and scanty infection of the true endothelial cells is comparable to their behavior in human tissues. Genetically, mesothelium and endothelium are closely related, but structurally and functionally they differ markedly. The above observations seem to show that they differ also in their resistance to infection by the typhus organism.

The apparently complete absence of *Rickettsiae* from both blood and tissue macrophages in the exudate was rather surprising. In tissue cultures, the *Rickettsiae* of Rocky Mountain spotted fever are found in

phagocytic cells (Wolbach and Schlesinger (6)) but typhus *Rickettsiae* have not been shown to infect these cells. Experiments now in progress in this laboratory indicate that typhus *Rickettsiae* grow luxuriantly (for a time) in mesothelial cells *in vitro*, but are never found in phagocytic cells.

The above observations suggest very strongly that typhus *Rickettsiae* are likewise incapable of multiplying in fibroblasts. It is of course impossible to be sure that the fibroblasts in our material were adequately exposed to infection. Tissue culture work now in progress will, we hope, answer this question definitely.

Study of *Rickettsia prowazeki* in sections has strengthened its position as an obligatory intracellular parasite. In smears one often finds large numbers of diffusely scattered extracellular organisms, but in view of the observations made on sections it seems necessary to conclude that these organisms are spilled from cells which are ruptured in the process of making the smear.

The observation that the *Rickettsiae*-containing serosal cells are essentially non-phagocytic is in agreement with the recent views on the origin of the peritoneal phagocytes. Cappell (7) recently found the serosal cells slightly phagocytic for carmine particles after repeated injection and believed that they might give origin to a certain number of the peritoneal macrophages. The absence of *Rickettsiae* from the phagocytic cells and the absence of ink particles from the *Rickettsiae*-containing cells is regarded as evidence against the serosal origin of any important number of peritoneal phagocytes.

SUMMARY

1. A satisfactory method is described for the topographical study of *Rickettsia prowazeki* in sections of the scrotal sac of typhus-infected guinea pigs.

2. In such sections *Rickettsiae* are always intracellular.

3. Typhus *Rickettsiae* multiply luxuriantly in the serosal cells and produce great distention of these cells.

4. *Rickettsiae* may be found in small numbers in the endothelial cells lining the underlying capillaries in the testes and scrotum, but are not seen in perivascular macrophages, connective tissue cells, fat cells, smooth or striated muscle fibres or epithelial cells.

5. *Rickettsiae* are rarely phagocytosed in small numbers by polymorphonuclears but are never seen in lymphocytes, plasma cells or eosinophiles.
6. When infected mesothelial cells proliferate and desquamate, they rapidly lose their content of *Rickettsiae*.
7. The large mononuclear cells seen in smears of the scrotal sac exudate may be separated into two groups: (1) the serosal (mesothelial) cells which become heavily infected with *Rickettsiae* but which are not phagocytic for graphite ink and (2) the macrophages (phagocytic cells) which do not contain *Rickettsiae*.

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EXPLANATION OF PLATES

PLATE 22

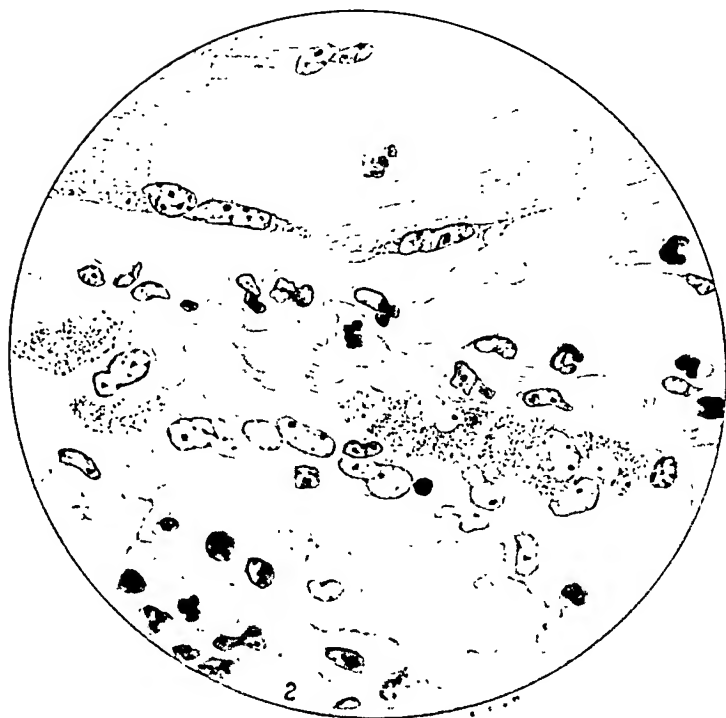
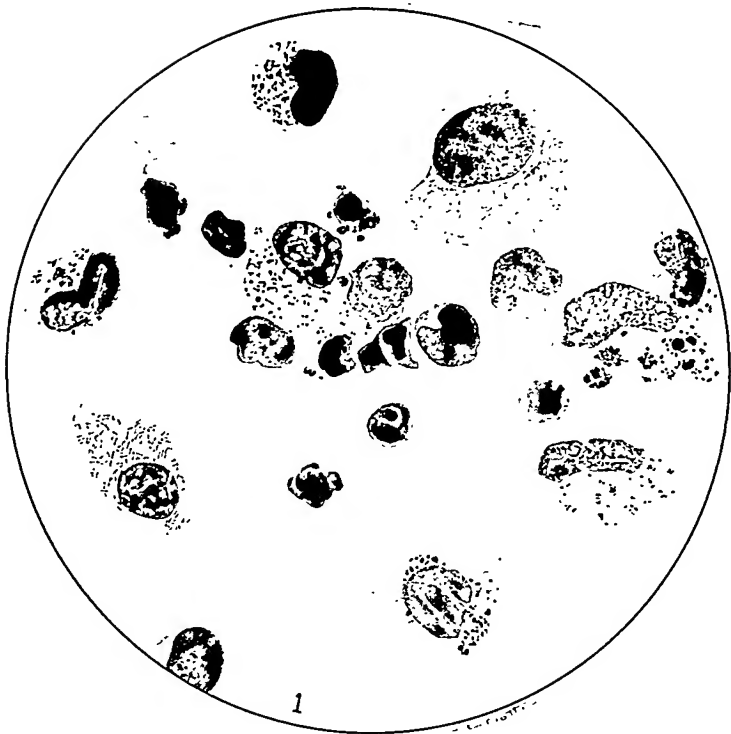
FIG. 1. Drawing of a representative field from a Giemsa-stained smear of scrotal sac exudate after intraperitoneal injection of graphite ink. One serosal cell (lower left) is lightly infected with *Rickettsiae* but has ingested no ink particles. Several macrophages are present with ingested ink particles but without *Rickettsiae*. $\times 800$.

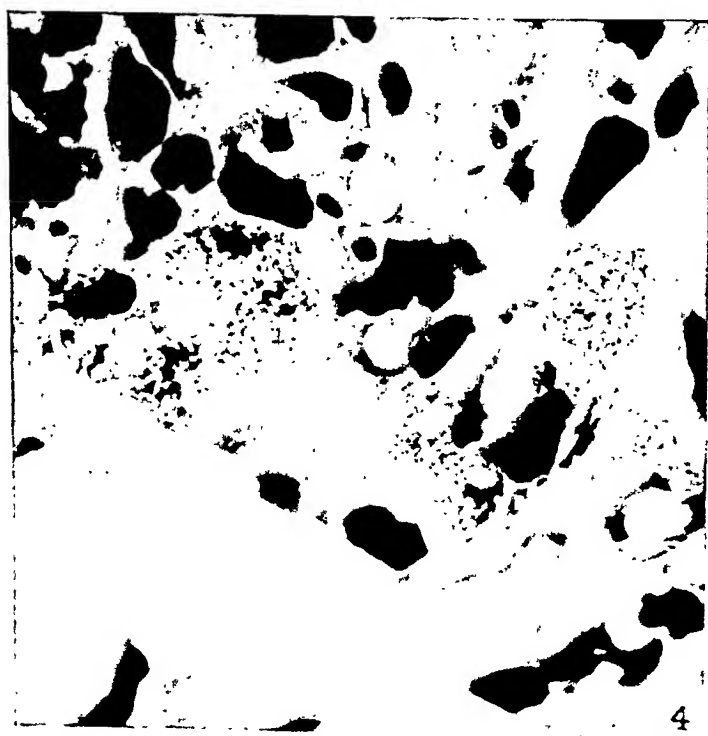
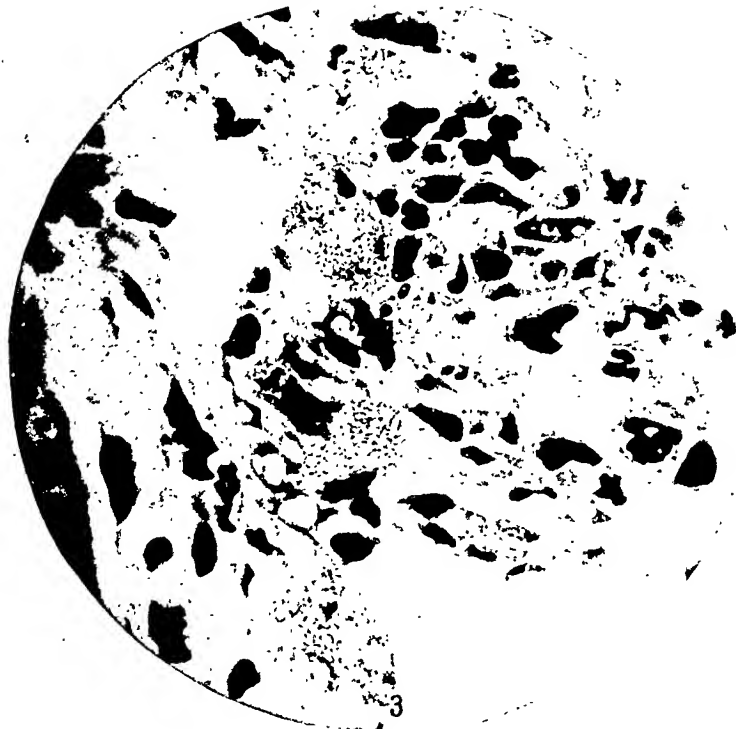
FIG. 2. Drawing of a representative field from a section of the scrotum and parietal tunica from a typhus-infected guinea pig. The majority of the serosal cells are heavily infected with *Rickettsiae*. Note the absence of *Rickettsiae* from the underlying cells and from the cells in the exudate. Regaud's fluid fixation. Giemsa stain. $\times 800$.

PLATE 23

FIG. 3. Photomicrograph showing a concentric whorl apparently formed by proliferation of serosal cells. Striated muscle of the scrotum is seen at the left. The row of cells occupying the position of the original serosa passes vertically through the center of the field. Note the *Rickettsiae* in these cells and the absence of *Rickettsiae* from the proliferated serosal cells at the right. $\times 789$.

FIG. 4. Higher magnification of a portion of the field shown in Fig. 3. Subserous collagen below. Layer of infected serosal cells crosses the center of the field horizontally. Proliferated serosal cells (without *Rickettsiae*) above. $\times 1578$.





ON PSYCHOPATHIC I.

THE EFFECT OF TESTICLE EXTRACT ON RED BLOOD CELLS IN VITRO

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The experiments of Duran-Reynals (1, 2) demonstrating that testicle extract, and, to a less degree, the extracts of certain other organs (kidney, skin, etc.) are endowed with the property of enhancing the lesions produced by vaccine virus and staphylococcus have been confirmed by McClean (3), Maitland (4) and others. Hoffman (5) and Pijoan (6) extended these results to many filtrable viruses and bacteria. The action of testicle extract is not clearly understood, although, since the first experiments of Duran-Reynals, it has appeared that the action is on the tissues of the animals and not directly on the vaccine virus itself. Duran-Reynals (1) has demonstrated that vaccine virus, when injected intravenously, localizes very readily in areas of skin previously injected with testicle extract. McClean (3) and Hoffman and Duran-Reynals (7) have shown that testicle extract increases the spread of inert substances (India ink) injected intradermally, probably by increasing the permeability of tissues and cells.

In the present paper some experiments are reported which were undertaken to test the view that the action of the effective factor in testicle extract is upon the host tissue. Since it would have been difficult to demonstrate directly any modification of the permeability of tissues into which the extract had been injected, an indirect method, allowing it to act on cells *in vitro*, has been chosen. Red blood cells were used for these experiments, since it is possible to observe very easily a modification of the permeability of their membranes by means of the so called "fragility test."

Some preliminary tests were made by mixing rat testicle extract and rabbit red blood cells, washing the latter and then adding them to graded solutions of sodium chloride. It was found that the fragility of the cells was considerably increased. Later a series of experiments was carried out, in which the action of testicle extracts on red blood cells of various species was tested.

* Fellow of the Rockefeller Foundation.

Method

All experiments were carried out according to the following technique:

Testicle and spleen extracts from rat, rabbit and guinea pig were prepared by grinding the organs with sand in a mortar, and adding an equal weight of 0.9 per cent salt solution. The resulting pulp was immediately centrifuged and only the supernatant fluid was used. Red cell suspensions were prepared as usual by centrifuging the defibrinated blood, aspirating off the serum and washing the cells three or four times in normal saline solution. After the last centrifugation, the preparations were brought to the original volume by the addition of normal saline solution. Both extract and red blood cells were used immediately, or at latest on the following day after remaining overnight in the ice box.

Each experiment was performed by mixing 1 cc. of red cell suspension with 1 cc. of testicle extract. The mixture was incubated for 2 or 3 hours at 37°. As control a mixture of red cell suspension and normal saline solution was kept for the same length of time in the incubator. The mixtures were centrifuged and the supernatant fluid aspirated off. The red cells were then washed with saline solution and, after centrifugation, brought to 2 cc. with it. A drop of the suspension was added to 1 cc. of saline solution at various concentrations, 0.90 per cent, 0.85 per cent, 0.80 per cent and so on down to 0.35 per cent. The control material was treated in the same way.

The amount of hemolysis of the red cells in each of the tubes was recorded, after they had been kept for about 30 minutes at room temperature and overnight in the ice box.

Experiments with Testicle Extract

In the first set of experiments, testicle extracts from rat, rabbit and guinea pig were tested against the red cells of each of these three species. The results with rat testicle extract are shown in Table I.

Table II shows the result of the experiments with rabbit testicle extract.

The results of the experiments with guinea pig testicle extract are shown in Table III.

Tables I, II and III show that testicle extract considerably increases the fragility of red cells. The cells of each species are not, however, sensitive in the same degree to the action of testicle extract.

These tests have been repeated several times, and the same results have always been obtained. It will be noted from these tables that the rat cells are little affected by any of the testicle extracts,

TABLE I

[illegible]

whereas guinea pig cells are greatly affected by all. Rat testicle extract is the most active in increasing fragility, and guinea pig extract the least active. In other words, within the limits of our observations, the species giving the most active testicle extract has the least sensitive red cells. The following table summarizes the action of the various testicle extracts against the several kinds of red cells.

TABLE IV

Testicle extract	Red blood cells		
	Rat	Rabbit	Guinea pig
Rat.....	±	++	++++
Rabbit.....	±	+	+++
Guinea pig.....	—	+	++

TABLE V

Hemolysis Induced by Rat Spleen Extract

Salt concentration, per cent....	0.90	0.85	0.80	0.75	0.70	0.65	0.60	0.55	0.50	0.45	0.40	0.35
Red cells of rat + S. E.	—	—	—	—	—	—	+	++	++	+++	++++	++++
Control	—	—	—	—	—	—	—	—	—	++	+++	++++
Red cells of rabbit + S. E.	—	—	—	—	—	—	—	+++	++++	++++	++++	++++
Control	—	—	—	—	—	—	—	±	+++	++++	++++	++++
Red cells of guinea pig + S. E.	—	—	—	—	—	—	—	—	—	++	+++	++++
Control	—	—	—	—	—	—	—	—	—	+	+	+++

The action of rat testicle extract against guinea pig red cells is so intense that a direct hemolysis of the cells has consistently been found after incubation. Such hemolysis has only occasionally been observed, and then only in a minor degree, in the case of mixtures of rabbit or guinea pig testicle extract with guinea pig red blood cells.

Experiments with Spleen Extract

The experiments of Duran-Reynals (1) have shown that extracts of several mesenchymatous organs and tissues yield results the opposite

of those with extracts of testicle. Instead of enhancing, they either do not modify or they inhibit infection. Spleen extract was notable in this respect, and hence the effect of spleen extract on red cells has

TABLE VI
Hemolysis Induced by Rabbit Spleen Extract

Salt concentration, per cent.....	0.90	0.85	0.80	0.75	0.70	0.65	0.60	0.55	0.50	0.45	0.40	0.35
Red cells of rat + S. E.	—	—	—	—	—	—	—	—	—	++++	+++++	+++++
Control	—	—	—	—	—	—	—	—	—	++	+++	++++
Red cells of rabbit + S. E.	—	—	—	—	—	—	—	—	++++	+++++	+++++	+++++
Control	—	—	—	—	—	—	—	—	+	+++++	+++++	+++++
Red cells of guinea pig + S. E.	—	—	—	—	—	—	—	—	—	++	+++	+++
Control	—	—	—	—	—	—	—	—	—	±	+++	+++

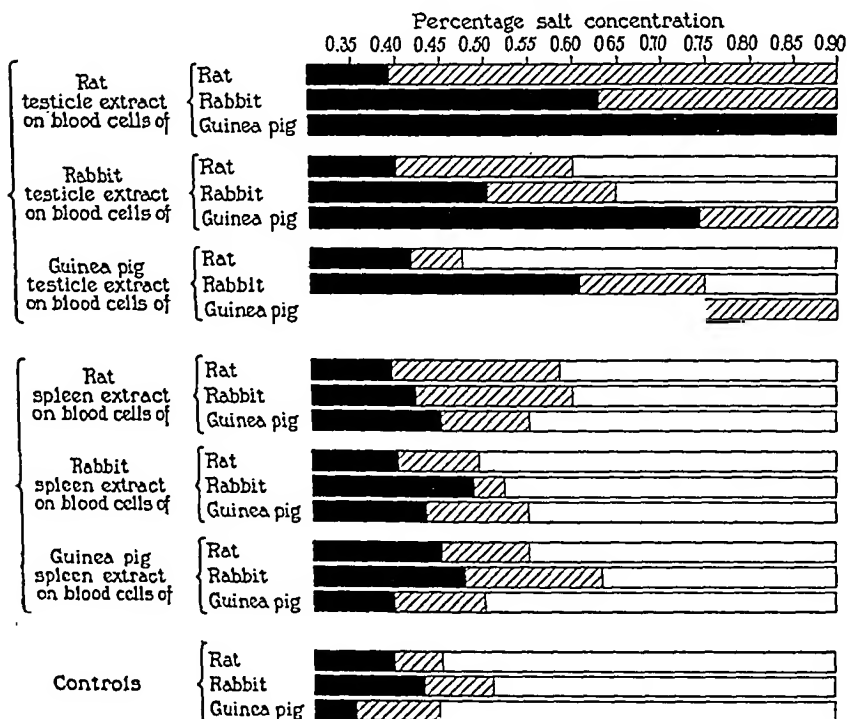
TABLE VII
Hemolysis Induced by Guinea Pig Spleen Extract

Salt concentration, per cent.....	0.90	0.85	0.80	0.75	0.70	0.65	0.60	0.55	0.50	0.45	0.40	0.35
Red cells of rat + S. E.	—	—	—	—	—	—	—	—	++	+++	+++++	+++++
Control	—	—	—	—	—	—	—	—	+	++	+++	+++
Red cells of rabbit + S. E.	—	—	—	—	—	—	+	+	+	+++++	+++++	+++++
Control	—	—	—	—	—	—	—	+	+	+++	+++++	+++++
Red cells of guinea pig + S. E.	—	—	—	—	—	—	—	—	—	+	+++	+++++
Control	—	—	—	—	—	—	—	—	—	—	+	+++

special interest. Several experiments have been carried out by the same technique used in the testicle extract experiments.

Tables V, VI and VII show the results of the first series of experiments with spleen extracts.

The results of these experiments, as shown in Tables V, VI and VII, indicate in a general way that the action of spleen extract on red cells is very much less than that of testicle extract. Each of the experiments has been repeated four times, and the results have been consistent. Text-fig. 1 shows the average degree to which the limit of



TEXT-FIG. 1. Average of the results of the experiments showing the effect of testicle and spleen extracts on the fragility of red blood cells of rat, rabbit and guinea pig. Shaded areas represent complete hemolysis; cross-hatch, partial hemolysis; blank spaces, absence of hemolysis.

fragility is brought down by the action of testicle and spleen extracts, as indicated by the maximum salt concentration at which hemolysis is produced.

In addition to the foregoing, another experiment was carried out to test the action of rat testicle extract against the red blood cells of three

normal human individuals. The extract sharply lowered the corpuscular resistance in all three cases.

In yet a third experiment with rabbit and rat red cells the effect of a purified bull testicle extract, which had proved very active in causing a spread of India ink through the subcutaneous tissue, was tested. The same lowering of corpuscular resistance was noted.

Finally we endeavored to detect a possible effect of rat testicle extract on guinea pig blood *in vitro*, by injecting the extract intravenously and comparing the normal resistance of the red cells with their resistance 2, 3 and 5 hours after the injection. No difference could be demonstrated. Negative results were also obtained when sheep blood cells, after a contact of 1 hour with rat testicle extract, were added to fresh guinea pig serum, or, conversely, when they were added to rat testicle extract after having been sensitized by the specific amboceptor.

DISCUSSION

It can be concluded from these experiments that testicle extract definitely increases red cell fragility, this action being more or less intense according to the animal species to which either testicle or red cells belong. Whatever the mechanism of any process of hemolysis, in the final analysis it is always through a modification of the permeability of the red cells that hemoglobin escapes into the surrounding liquid. Testicle extract has plainly the power *in vitro* to increase this permeability. The observation gains in significance when considered in the light of previous reports (1, 2, 5, 6), showing that testicle extract increases tissue permeability. That the factor which enhances the activity of infectious agents, the so called Reynals factor, is responsible for these effects has yet to be proved. But all the evidence thus far collected is in line with the view that it is responsible, and that the Reynals factor enhances the effects of viruses and bacteria through the local changes in cell permeability that it brings about in the tissues of the host.

The fact that spleen extracts have little or no power to lower the level of red cell resistance is consonant with the observation of Duran-Reynals (1) that these extracts fail to enhance the infectivity of

viruses and bacteria, to increase the spread of inert particles through the tissues.

Many authors have been interested in whether the spleen is endowed with hemolytic power. The problem is important from the point of view of the pathogenesis of certain forms of anemia and jaundice, and also in relation to the much debated question of the origin of blood complement. A hemolytic power of the spleen has yet to be conclusively demonstrated. Korschum and Morgenroth (8), Donath and Landsteiner (9), Metschnikoff (10), Achard, Foix and Salin (11) and others have found in splenic extracts but a very slight hemolytic power, no greater than that shown by various other organs. However, the technique generally used in these experiments was simply to mix spleen extracts and red cells and to observe whether or not hemolysis occurred. In our experiments spleen extracts were found to have only a very slight power of lowering corpuscular resistance.

Others have noted—and we can confirm the observation—that occasionally spleens are found which exhibit a marked hemolytic power. Whatever the reason for this power, it does not compare with that consistently shown by testicle extracts.

CONCLUSIONS

Testicle extract possesses a notable power to increase red cell fragility. Testicle extracts of the rat, rabbit and guinea pig all exhibit this property, which is most pronounced with the extract from the rat and least so with that from the guinea pig.

Splenic extract does not possess the property, or possesses it only to a very slight degree.

These results support the hypothesis that the factor in testicle extract which enhances infections (the Reynals factor) does so by altering the permeability of the host tissue.

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PYOCYANINE, AN ACCESSORY RESPIRATORY ENZYME

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In a previous study (1) it has been shown that pyocyanine in mixture with its leuco compound is a reversible oxidation-reduction system that at $\text{pH} > 6$ behaves like any one of the known reversible dyestuffs of quinoid structure. How this property may manifest itself under biological conditions is shown in the following experiment:

A culture of *Bacillus pyocyaneus* in which the blue pigment has been formed becomes colorless on the exclusion of air. On agitation with air the blue color is restored. The bacteria reduce the pigment to the colorless leuco base, which then by the oxygen of the air is re-oxidized to the colored dye. Pyocyanine acts here as an autooxidizable acceptor for labile hydrogen of the microorganisms. This experiment shows that pyocyanine may play a rôle in dehydrogenative oxidation of bacteria in that it undergoes a cycle from the oxidized to the reduced and back to the oxidized form. As potentiometric studies (1) have shown that this cycle is also reversible thermodynamically, *i.e.*, in the course of this cycle the entropy of the system, pyocyanine-leucopyocyanine, remains constant, all the theoretical requirements are given which would allow pyocyanine to serve as an ideal catalyst for bacterial oxidation processes. But before pyocyanine can be so considered, proof is required that this pigment actually accelerates the oxygen consumption, *i.e.*, the respiration of bacteria. This proof is submitted in what follows:

Outline of the Experiment

The Warburg manometric method was applied to measure the respiration of *pyocyaneus* with and without the addition of pure recrystallized pyocyanine, of bacilli that had been cultivated under conditions impeding the formation of pigment and that had been washed free from all traces of nutritive media and suspended in a phosphate buffer.

Procedure.—The following strains of the American Type Culture Collection were used: (1) *Pseudomonas aeruginosa* No. 97, Army Medical School, Washington, D. C.; and (2) No. 256 E. O. Jordan, University of Chicago (Catalogue of Cultures, 1928). The two strains behaved in every respect identically. Cultures free from pigment, or producing only traces of the green fluorescent pigment were obtained at 30°C. in Blake bottles on lemco agar (0.3 per cent lemco, 0.5 per cent NaCl, 1 per cent Witte peptone, 2 per cent agar, pH 7.6). Cultures 15 hours to 5 days old were used.

The bacteria were swept off the medium and washed three times in the centrifuge with distilled water, and finally suspended in phosphate buffer (Sørensen). The suspension was made thoroughly homogeneous by filtration through paper and by shaking in a machine. A count of the bacterial suspension was not made as all measurements were comparative and hence the absolute quantity of bacteria was irrelevant. Therefore not the measured absolute values of respiration, but only the relative increases, are comparable for different experiments. 1 to 2 cc. of the bacterial suspension were placed in the main compartment of Warburg's micro-respiration vessels. The small inner vessel was filled with 0.2 cc. of 6 per cent NaOH. Pyocyanine was purified by recrystallization out of chloroform and stocked in N/20 HCl. Before each experiment the necessary amount of stock solution was adjusted to the proper pH (around 7.0) by the addition of secondary sodium phosphate. This dye solution was then added to the bacterial suspension in the proportion of about 1 to 10. The final molar concentration of pyocyanine was 1/5,000 M in all experiments. All the work was carried out under aseptic conditions. Aerobic and anaerobic test cultures produced at the end of the experiments showed, firstly, that the bacilli were still living and capable of reproducing, and, secondly, that no contamination with foreign microorganisms had occurred.

RESULTS

Effect of Pyocyanine on the Respiration of B. pyocyaneus.—All experiments showed unequivocally that the bacilli, suspended in an indifferent phosphate buffer, consume a small but definitely measurable amount of oxygen. Addition of pyocyanine increases this amount up to many hundred per cent as can be seen in Table I.

Fig. 1 represents the results of a typical experiment. Control tests prove that under the conditions pyocyanine itself does not consume any oxygen. Only at strongly alkaline reaction, beyond pH about 9.4, does pyocyanine oxidize spontaneously and irreversibly.

Effect of Temperature.—The figures given above were found at temperatures ranging from 18–24°C. Elevation of temperature up to 37°C. increases the basic oxygen consumption of the bacteria alone.

The increase brought about by pyocyanine over this already increased respiration, is not so large as at the lower temperatures, but is still quite important: 29 per cent to 127 per cent.

Effect of pH.—Fig. 2 shows that the increase of respiration induced by pyocyanine is about constant between the tested limits of pH, 5.3

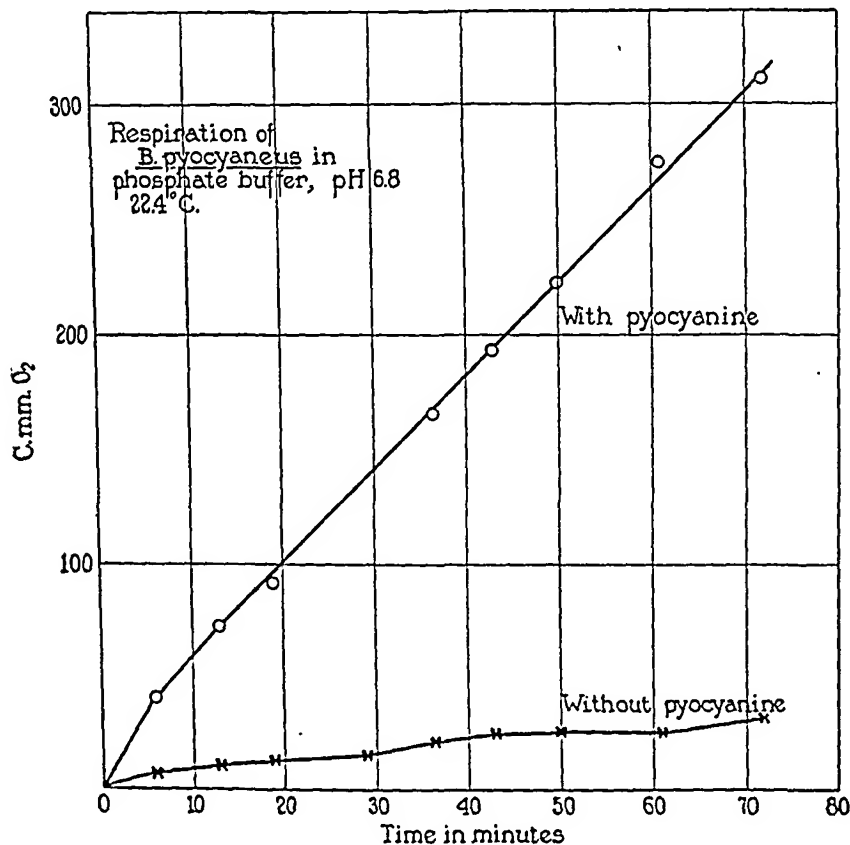


FIG. 1

and 7.7. The increase of the pyocyanine respiration observed between pH 5.3 and 7.2 parallels approximately the increase in the basic respiration of the bacteria alone.

The Oxygen Consumption of Killed Bacilli.—Does the excess consumption of oxygen brought about by pyocyanine really signify an

increase in respiration of the living bacteria or does the extra oxygen serve only for the oxidation of some bacterial product, in the sense of

TABLE I

Oxygen Consumption of Washed B. pyocyaneus Suspended in Phosphate Buffer

No.	pH	Temp.	Oxygen consumed		Increased respiration
			With pyocyanine	Without pyocyanine	
		°C.	c.mm.	c.mm.	per cent
1	7.4	21.4	— 4	— 51	1,275
2	7.4	21.4	— 4	— 41	1,025
3	6.8	22.3	— 32	— 310	970
4	6.8	22.2	— 527	— 290	550
5	6.9	20.9	— 4	— 96	2,400

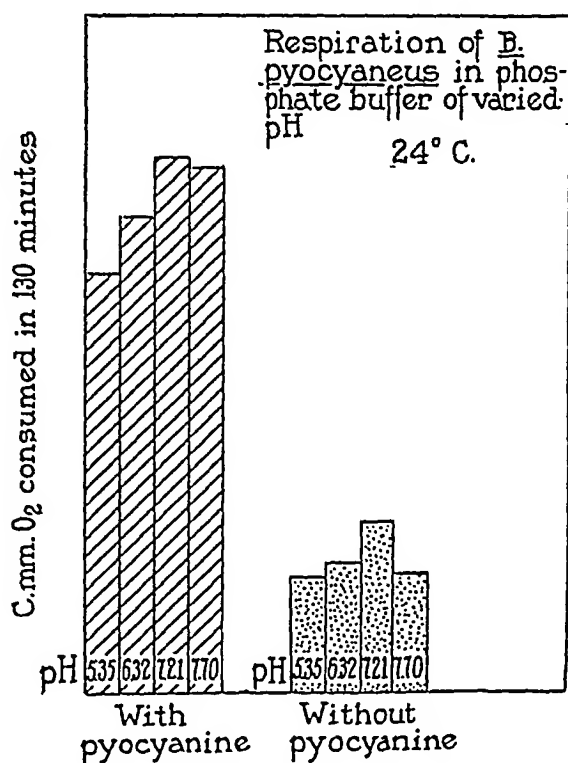


FIG. 2

the accessory respiration (*Nebenatmung*) of Batelli and Stern (2)? Bacteria killed by 1 hour exposure to 56°C., or by the addition of tolu-

ene did not show any oxygen consumption at all, either with or without pyocyanine (see Fig. 3). Therefore it may be concluded that pyocyanine affects actually the respiration (*Hauptatmung*) of the living bacteria.

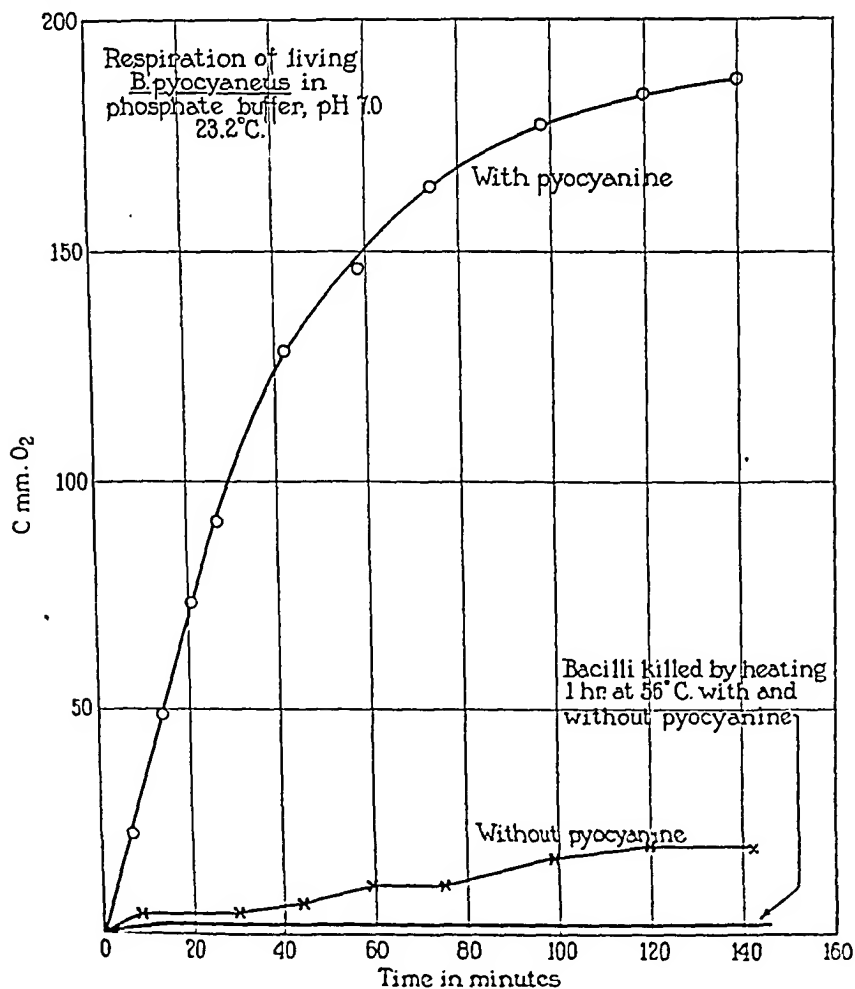


FIG. 3

Effect of Pyocyanine on the Respiratory Quotient.—Is the increase of respiration induced by pyocyanine purely quantitative or does pyocyanine affect also the process of oxidation qualitatively? With a view

to elucidating this question the respiratory quotient was determined by the method of Warburg and Yabusoe (3). As shown in Table II, it was found that without pigment and even when appreciable amounts of oxygen are consumed, the formation of carbon dioxide is very small; but it is enhanced by the addition of pyocyanine to such extent that the respiratory quotient increases many times and approaches 1.0. It appears thus that the oxidations brought about by the bacteria without the pigment are to a great extent incomplete, but that they can be completed with its assistance.

Of What Substances Is the Oxidation Catalyzed by Pyocyanine?—Since in all the experiments described above the bacteria were freed through washing of all traces of the nutritive media, only such substances as are part of the microorganisms can come under consideration. This conclusion is sustained by the analysis of the curves show-

TABLE II

Respiratory Quotient of Washed B. pyocyanus Suspended in Phosphate Buffer

With pyocyanine	Without pyocyanine	Temp.	pH
		°C.	
0.77	0.17	22.2	6.8
0.9	0.1	20.9	5.8

ing the oxygen consumption as a function of time. As Fig. 3 shows, the curve for oxygen consumption under the influence of pyocyanine progresses during the 1st hour in a straight line, *i.e.*, the consumption is directly proportional to time, but thereafter it falls off more and more, the curve tending to become parallel to the abscissa. But, in the presence of an assimilable substance, such as glucose, asparagin, or pyruvic acid, the curve of oxygen consumption continues to progress in a straight line (Fig. 4). It follows that the flattening of the curve just mentioned cannot be caused by a diminution of the partial oxygen pressure, but is the consequence of an exhaustion of oxidizable substances. Furthermore it cannot be that the oxidation of these added substances is catalyzed by pyocyanine because if they are added without pigment to the washed bacteria, the respiration also increases many times. Under such circumstances the subsequent addition of

pyocyanine does not appreciably increase the respiration which has already been enhanced. This falls in with the findings on the influence of higher temperature reported in a previous paragraph. It may

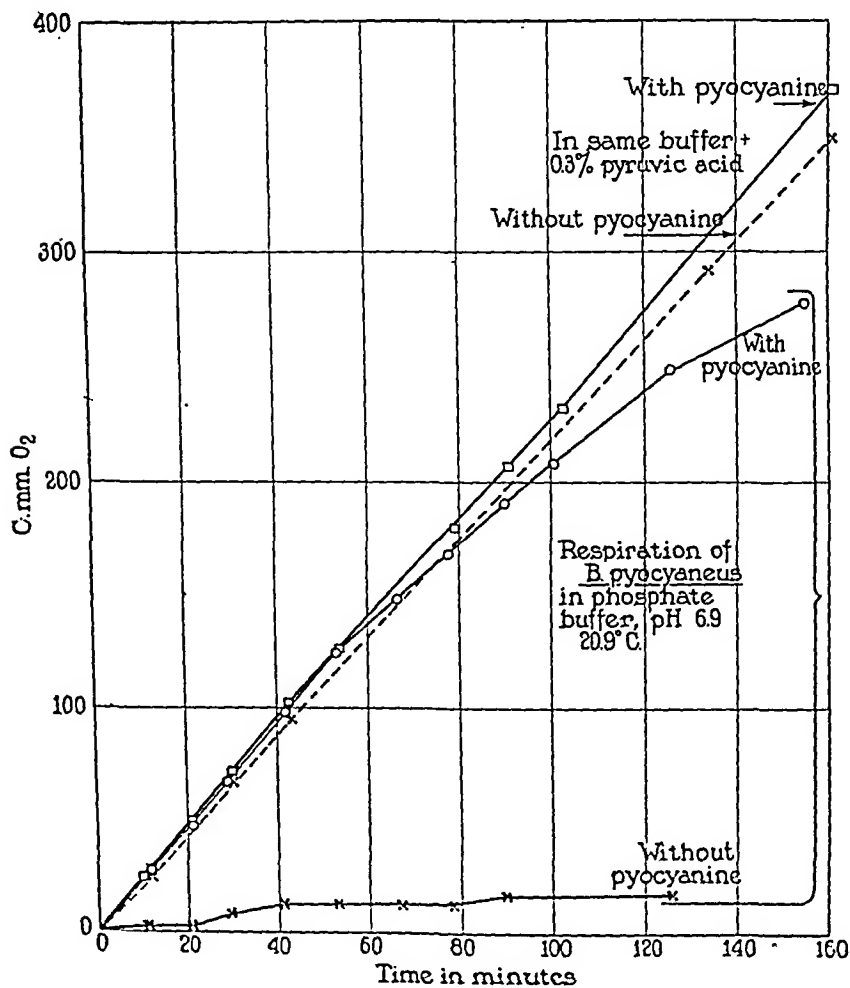


FIG. 4

be concluded that pyocyanine does not enhance respiration under all circumstances, but that it only catalyzes the oxidation of certain unknown substances closely associated with the bacteria, perhaps bac-

terial lipoids and polysaccharides, or certain products of their disintegration.

The Significance of Pyocyanine as a Respiratory Enzyme.—The fact that *B. pyocyaneus* has an appreciable oxidative metabolism even in the absence of pyocyanine tends to indicate that the latter does not play the main rôle in the respiration of the bacteria. Also further experiments show that pyocyanine is an accessory ferment, the effect of which is dependent upon the presence of one or several other respira-

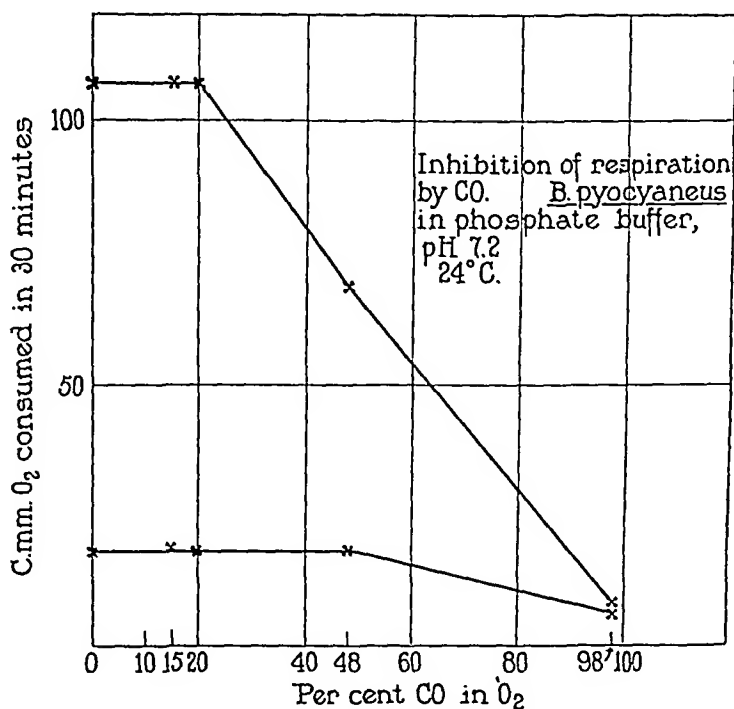


FIG. 5

tory ferments, amongst which must be considered the cytochrome shown spectroscopically by Yaoi and Tamiya (4) to be present in *B. pyocyaneus*. Their observation has been fully confirmed in the course of these experiments.

In a test-tube experiment at pH > 5, the oxidation and reduction of leucopyocyanine is in no way impeded by carbon monoxide or by potassium cyanide. But, as shown in Figs. 5 and 6, these substances, known readily to form complexes with heavy metals, impede not only the basic respiration of the bacteria alone, but also the respiration due

to pyocyanine. Figs. 5 and 6 further reveal the curious phenomenon that the pyocyanine enhanced respiration is affected at a lower potassium cyanide and carbon monoxide threshold-concentration than is the basic respiration. As the association of different hemochromogens, known as cytochrome, does not combine with carbon monoxide

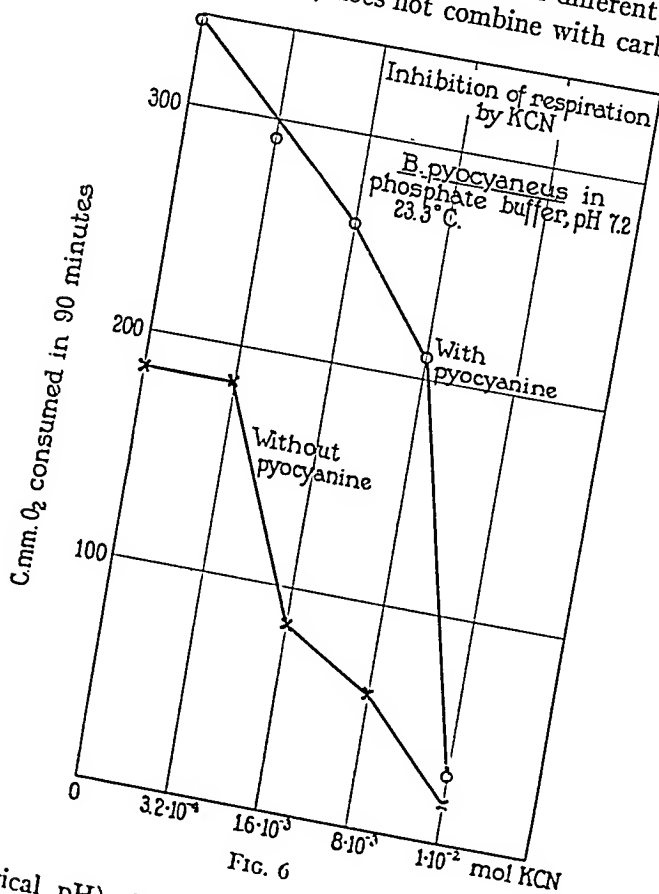


FIG. 6

(at physiological pH) the observation that the respiration of *B. pyocyanus*, with and without pyocyanine, is impeded by carbon monoxide indicates that the cytochrome, although it may be a necessary link in the chain of bacterial oxidation catalysts, is not the final one, and that that chain is not complete without the carbon monoxide sensitive respiratory enzyme of Warburg.

The increase of respiration induced by pyocyanine is not specific for the species *B. pyocyaneus*. It has been observed in varying degree with other microorganisms containing cytochrome, *i.e.*, with staphylococci (Fig. 7) and pneumococci (Fig. 8); whereas with obligatory

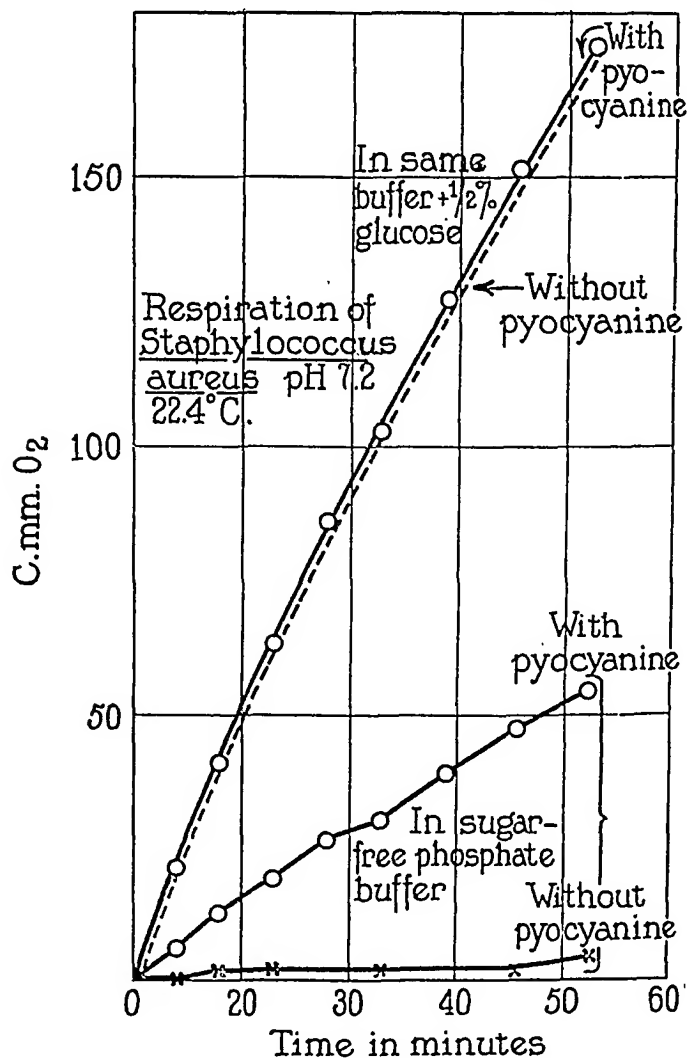


FIG. 7

anaerobics such as *B. tetani*, which do not contain any cytochrome, pyocyanine had no effect. Furthermore, its effect is not restricted to bacteria only, but may be observed also with animal cells. Fig. 9 shows the increase in respiration induced by pyocyanine in red blood corpuscles of the rabbit.

The fact that pyocyanine is to a large degree not species-specific makes reasonable the view that the characteristic properties which condition its significance as an oxidation catalyst are of a purely physi-

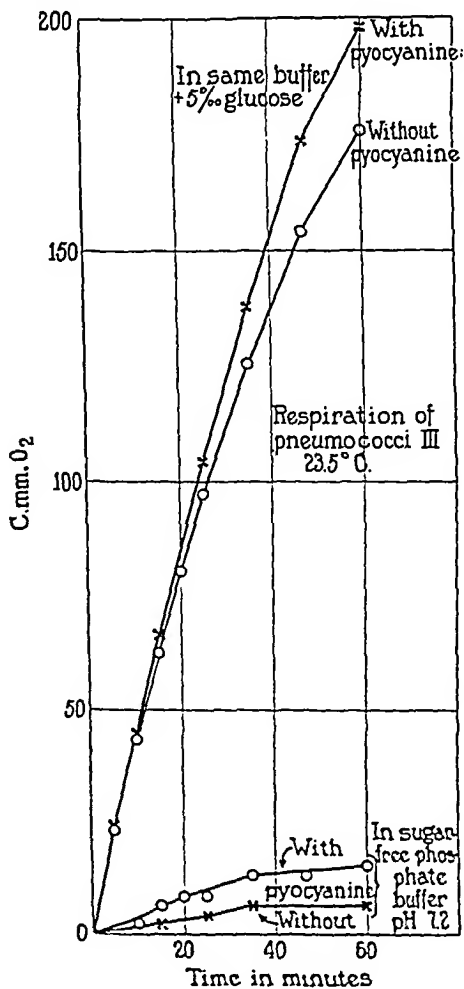


FIG. 8

cochemical nature, the decisive feature being reversibility. If this is so one may expect that any other reversible system of the same potential range would have the same biological effect, granting that it has no other properties, such as solubility, permeating propensity,

toxicity, which might act as counteracting influences. Pyocyanine seems to be favored in respect to these secondary chemical properties by its low affinity for proteins and its high affinity for lipids. Of the

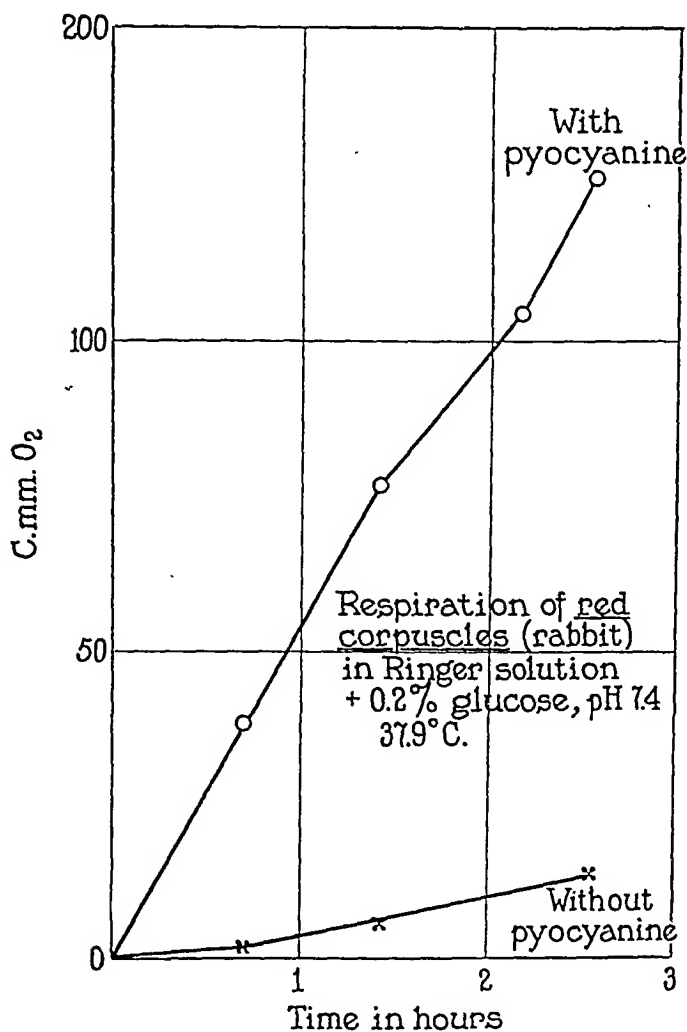


FIG. 9

synthetic organic oxidation-reduction systems methylene blue and indigo tetrasulfonate are closest to pyocyanine in their potential range. At pH 7.0 and 30°C. the normal potentials are:

Indigotetrasulfonate.....	ms. -46
Pyocyanine.....	-34
Methylene blue.....	+11

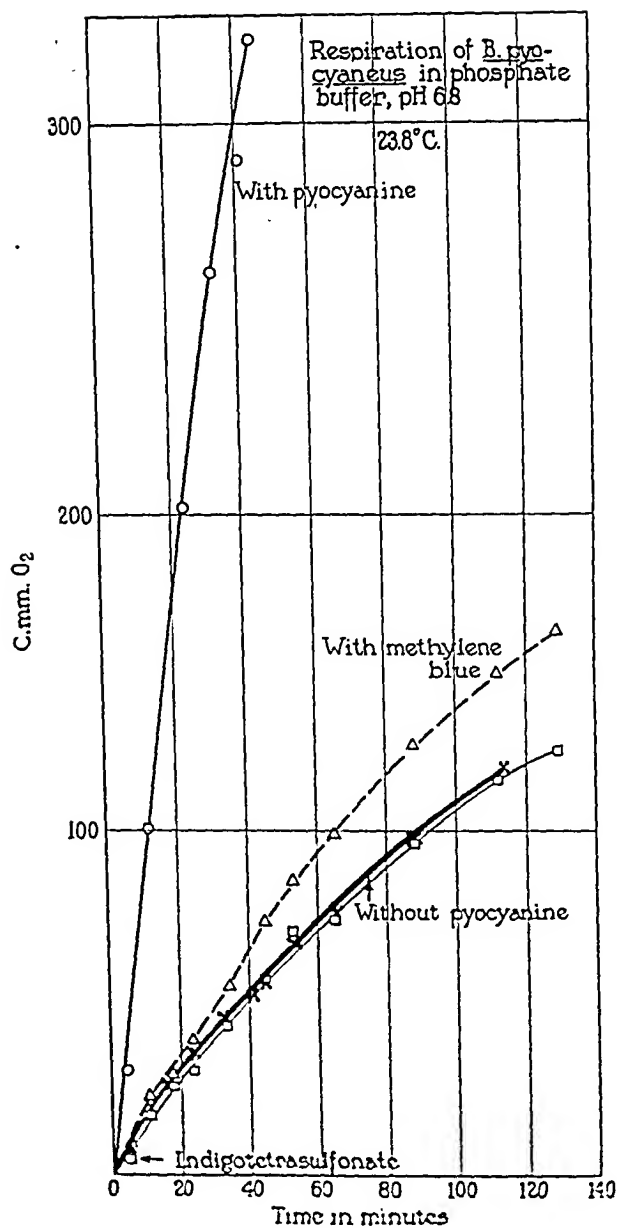


FIG. 10

Fig. 10 shows the comparative effect exerted by these three substances in equal molecular concentrations ($\frac{\text{Mol}}{5,000}$) on the respiration of *B. pyocyaneus*. Whereas indigotetrasulfonate with a potential range more negative than that of pyocyanine is devoid of effect the more positive methylene blue induces an increase of 30 per cent, and pyocyanine itself an increase of 440 per cent. In connection with the significance to be attributed to the value of the normal potential of the catalyst there is one obvious condition: It must be more positive than the reduction potential of the compound of which it catalyzes the oxidation. The findings just given would seem to indicate that the potential range of pyocyanine represents the lower limit of the potential range in which hydrogen can be accepted from the bacteria.

The experiment with methylene blue links this work with earlier observations showing that methylene blue enhances not only the respiration of acetone-treated yeast and staphylococci (Meyerhof (5)), but also the respiration of living cells as *e.g.* red and white blood cells, sea urchin and star fish eggs, and cancer cells (Barron and coworkers (6)). These observations disclosed the possibility of biological oxidations being catalyzed by autooxidizable hydrogen acceptors. The search for substances in the living cell which act like the artificial model, methylene blue, led to the isolation of two kinds of substances; First, a group having the character of reversible systems (echinochrome, hermidin) but not as yet proved to play an actual rôle as respiratory enzymes of the living cell; second, the respiratory supplement discovered by Michaelis and Salomon (7) in extracts of various organs, especially liver, which has a mode of action not yet shown to be that of a reversible system.

SUMMARY

Pyocyanine, the blue pigment of *B. pyocyaneus*, can increase the respiration of living cells to a great degree (maximum observed increase 24-fold). The reversibility of its oxidation and reduction is responsible for this. The effect is non-species-specific and has been observed in varying degrees with *B. pyocyaneus*, *Staphylococcus aureus*, *Pneumococcus* Type III, and the red blood corpuscles of rabbits.

The effect of pyocyanine is dependent on the presence of another

respiratory ferment sensitive to potassium cyanide and carbon monoxide.

The increase of respiration induced by pyocyanine is paralleled by an increase in the respiratory quotient. The pyocyanine catalysis is not indiscriminately effective in all oxidations, but only in the oxidation of certain substances closely associated with the bacterial body.

I wish to express my sincere thanks to Dr. L. Michaelis, in whose laboratory this work was carried out, for many helpful suggestions and criticisms.

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BLOOD CHEMICAL CHANGES IN EXPERIMENTAL STREPTOCOCCUS SEPTICEMIA

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Studies in the chemical changes in the blood of animals experimentally infected with virulent *Streptococcus hemolyticus* have seldom been made. Dragstedt (1) determined the actual pH of the blood of four rabbits which had been given large injections (2 to 4 cc.) of cultures of this organism intraperitoneally. In the two animals which died the pH fell from 7.7 and 7.6 to 7.5 and 7.3 respectively. The animals which recovered showed actual increases in acidity to the same degree, leading one to suppose that this factor could not have been of outstanding importance as a cause of death. Hirsch (2) injected various types of bacteria, including a hemolytic streptococcus, into rabbits by the intravenous route. With this microorganism he found a lowering of the CO₂ capacity to 48 volumes per cent after 5 hours, followed at 24 hours by an alkalosis (80 volumes per cent) and normal figures throughout the following 6 days. It is not stated whether the streptococcus infection was fatal for the rabbit. In a later communication (3) he again reported the results of a similar injection into a rabbit. Here the CO₂ capacity fell from a preliminary figure of 59 volumes per cent to 54 volumes at 5 hours, and remained normal thereafter. The blood sugar concentration of this animal was increased, rising from 106 mg. at the time of infection to 135 mg. at 5 hours, 120 mg. at 23 hours, and 141 mg. at 29 hours. The streptococcus used is referred to as pathogenic. The data show, however, that the constituents studied were not much affected by the organism used.

The purpose of the work reported here was to ascertain the cause of death from streptococcus septicemia in rabbits, so far as it may be related to chemical changes. To this end the following constituents were studied: Blood sugar, liver glycogen, CO₂-combining capacity, inorganic phosphorus, calcium, non-protein nitrogen, and creatinine.

Material and Methods

The carbon dioxide-combining capacity and the creatinine were determined by the usual laboratory methods. For the other constituents the following techniques were employed: blood sugar (Folin's micro method (4)); glycogen (Sloss

(5)); inorganic phosphorus (Tisdall (6)); calcium (the Clark-Collip modification of the Kramer-Tisdall method (7)); non-protein nitrogen (Folin and Svedberg (8)).

Rabbits were used. Doses of a 24 hour broth culture of *Streptococcus hemolyticus*, varying in amount from 0.3 to 0.75 cc., were given in the ear vein. This organism is highly virulent for rabbits when given intrapleurally, as little as one ten-millionth of a cubic centimeter of a culture producing a rapidly fatal empyema. The same strain given intravenously is much less virulent, probably because when given by this route the organisms are brought directly in contact with tissues capable of destroying them. Hopkins and Parker (9), using a streptococcus virulent for rabbits, have shown that the lung and liver are particularly active in this destruction, and that only those microorganisms which lodge in the muscles continue to multiply. From the muscles they are washed out into the blood stream to give rise to the septicemia.

A total of seventeen animals was used in the experiments. They fall into two groups, depending upon the length of survival after infection. Those which died within 2 to 4 days are designated as having fulminating infections. The average length of survival in this group was 61 hours and the average dose 0.5 cc. Ten of the animals are included in this series. In the second group, which are designated as acute cases, the survival times averaged 6 days and the dose averaged 0.4 cc. The remaining seven animals are in this category. It is clear that the relationship between size of dose and survival time is by no means absolute.

About 12 cc. of whole blood are necessary to complete the tests named above. As it would be injurious to the animal to take this amount of blood at short intervals during the course of a severe infection, not all the tests were done on any one animal at a given time. As a result it is necessary to present the data as averages.

RESULTS

The results of the determinations are given in Figs. 1 to 4, and the averages upon which the curves are based are shown in Table I. The abscissae represent fifths of the period during which the disease ran its course. In this way the acute type of infection is foreshortened in relation to the fulminating type, but it was thought that any disadvantage arising from this method of representation would be compensated for by the greater ease in comparing the two curves.

Blood Sugar.—Fig. 1. The averages for this constituent show that in the fulminating group the blood sugar drops at a rather constant rate throughout the infection. In the group of acutely infected animals the drop does not begin until the disease has run about two-fifths of its course, but from that time on the glucose gradually lessens. Nevertheless, the levels reached in the terminal stages by both groups are not those at which a hypoglycemia can be considered present.

During the last part of the disease the rabbits do not eat, and it was considered possible that part of the fall in blood sugar might be accounted for in this way. The literature on the effect of starvation on rabbit blood glucose shows, however, that a curve such as this is not obtained in animals from which food is being withheld. On the contrary a constant level of blood sugar is maintained for several days,

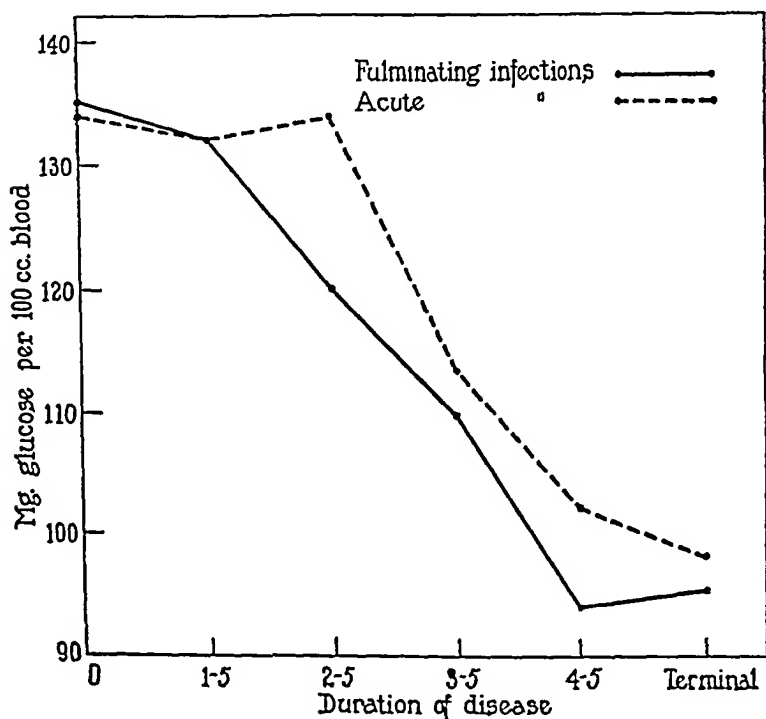


FIG. 1. Average concentration of blood sugar in the blood of rabbits with acute and fulminating infections with *Streptococcus hemolyticus*.

and the levels instead of decreasing steadily as in the streptococcus infected rabbits show increases and decreases throughout the period (Kisch, Simons, and Weyl (10)).

Liver Glycogen.—In six animals, two of the fulminating and four of the acute type, the liver glycogen was determined at death. In no case was glycogen absent, and expressed as glucose, it varied from 1 to 21.6 mg. per gram of liver, averaging 8.1 mg. for the series. Such a

result might of course be expected in view of the nearly normal levels of blood glucose at death, although it has been shown (Linton (11)) that in acute trypanosome infections in rats the liver glycogen disappears at a relatively long period before death, while the blood sugar remains at a normal level until the agonal stage, when a severe hypoglycemia is present.

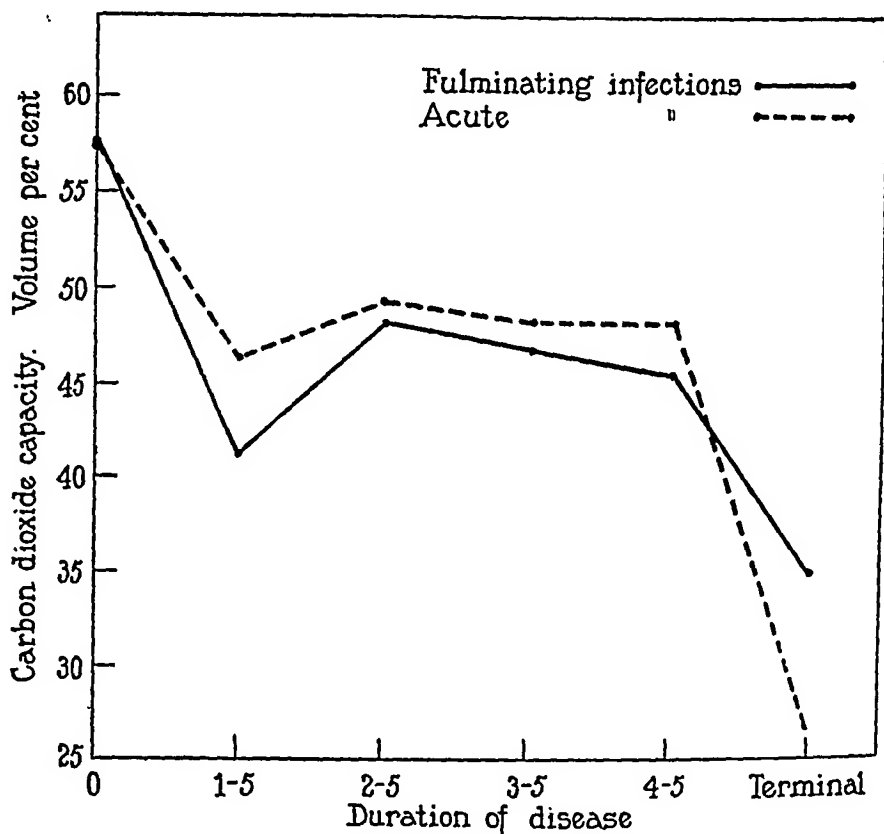


FIG. 2. Average carbon dioxide capacity in rabbits with acute and fulminating infections with *Streptococcus hemolyticus*.

Carbon Dioxide-Combining Capacity.—The curves of the CO_2 capacity are given in Fig. 2. In both groups an immediate drop occurs, to 46.0 volumes in the acute and to 41.5 in the fulminating cases, followed by a rise which brings the capacity back to the lower limit of the normal range of variation in the rabbit. This value persists through the remainder of the disease at a rather constant level, until the terminal stage is reached, when a second sharp

drop occurs. In the acutely infected animals the final value is 26.2 volumes and in the fulminating infections it averages 34.8 volumes per cent.

Inorganic Phosphorus and Calcium.—The data for these constituents are presented together in Fig. 3, since it seems well established that there is a reciprocal relation between them. The inorganic phosphorus

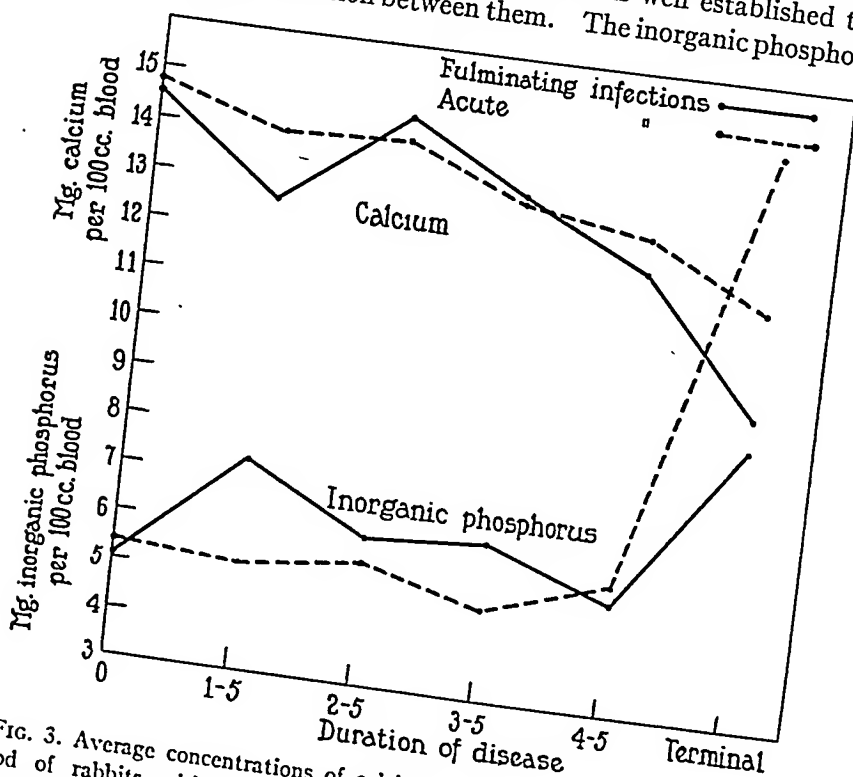


FIG. 3. Average concentrations of calcium and inorganic phosphorus in the blood of rabbits with acute and fulminating infections with *Streptococcus hemolyticus*.

in the acute cases shows a marked terminal rise, reaching an average level of 14.2 mg. per 100 cc. A similar terminal rise, although to a less marked degree, is shown in the fulminating infections. Here the average figure (8.6 mg.) approximates the upper limit of the normal variations (Harnes (12)).

The study of the calcium variations was undertaken because of the

symptoms of disturbance of concentration of this element. These include weakness of the limbs during the terminal portions of the disease and a considerable degree of hyperexcitability. The average figure for the fulminating group, 9.3 mg., shows a more severe change in this constituent in this group than in the acutely infected rabbits, where the terminal figure is 11.5 mg. While the general trend of the

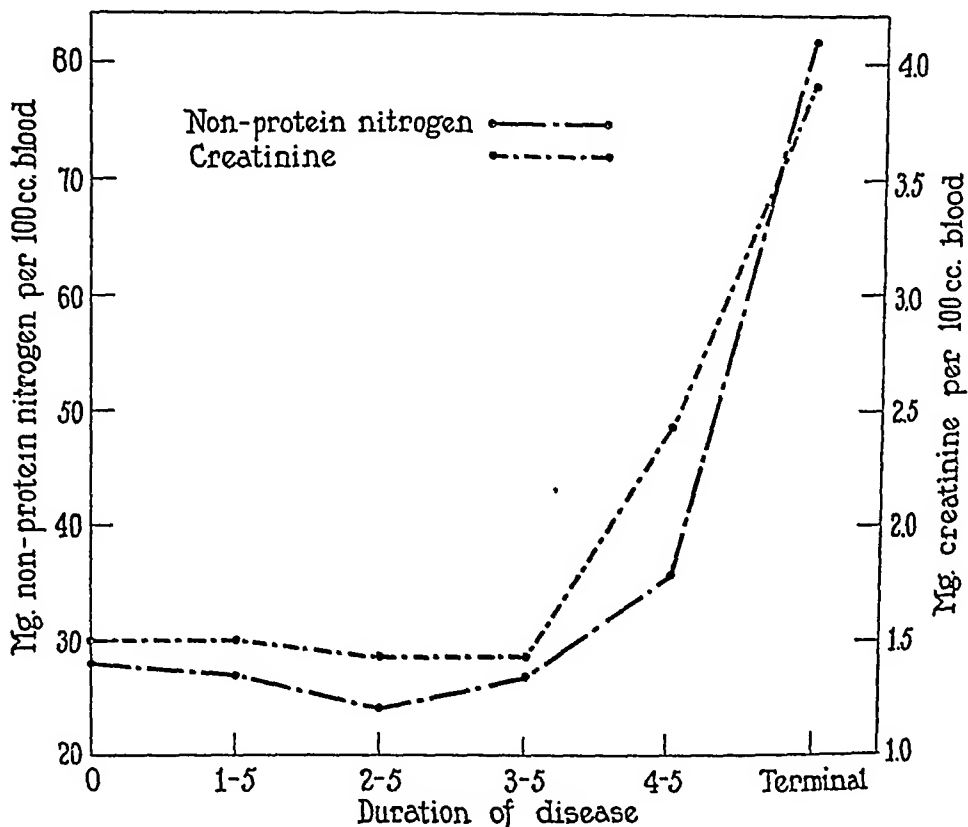


FIG. 4. Average concentrations of non-protein nitrogen and creatinine in the blood of rabbits infected with *Streptococcus hemolyticus*. Combined data from fulminating and acute cases.

curves is downward, it can only be concluded that the drop in calcium is a terminal phenomenon.

Non-Protein Nitrogen and Creatinine.—These two constituents were determined as measures of kidney function (Fig. 4). As the curves show they remain at normal concentrations in the blood until late in the disease, at which time they show marked rises, reaching

an average of 81.6 mg. for the non-protein nitrogen, and 3.9 mg. for the creatinine. The data for the fulminating and acute cases are combined in the chart, and given separately in Table I.

Histological Changes.—In sections of kidney taken from animals killed before the disease had reached its terminal stage (as shown by non-protein nitrogen and creatinine determinations), the histological changes were slight and consisted essentially of cloudy swelling of the

TABLE I
Changes in Averages of Blood Constituents in Fulminating and Acute Septicemias Due to Streptococcus hemolyticus

Duration of disease	Blood sugar		CO ₂ capacity		Inorganic phosphorus	
	Fulminating	Acute	Fulminating	Acute	Fulminating	Acute
0	135	134				
1-5	132	132	55.5	55.2		
2-5	120	132	41.5	46.0	5.1	5.4
3-5	110	134	48.3	48.9	7.2	5.2
4-5	94	117	47.3	48.3	6.0	5.6
Terminal	95	111	43.9	48.3	6.2	4.9
		101	34.8	26.2	5.2	5.6
					8.6	14.2
Duration of disease	Calcium		Non-protein nitrogen		Creatinine	
	Fulminating	Acute	Fulminating	Acute	Fulminating	Acute
0						
1-5	14.5	14.7				
2-5	12.6	13.9	28.1	27.1		
3-5	14.4	14.0	32.5	20.8	1.4	1.8
4-5	13.3	13.0	28.0	20.7	1.5	—
Terminal	12.0	12.7	23.9	27.5	—	1.4
	9.3	11.5	27.2	37.1	—	1.4
			61.9	96.3	4.2	1.5
					4.2	3.5

tubular epithelium. The glomeruli were normal. Kidneys taken at death, after the alkaline reserve had been exhausted, showed, on the other hand, a severe nephritis. The tubular epithelium was severely injured, the tissue was edematous and hemorrhagic, and the glomeruli were destroyed in part. It is evident that the severe histological changes are terminal and can be explained by the occurrence of an actual acidity of the blood. The histological changes are more severe in animals with acute infections than in those in which death occurs

in a briefer period. This difference may be due to the longer continuation of the stage of uncompensated acidosis. .

DISCUSSION

It is evident that the results obtained may be explained on the assumption of a large amount of acid production *in vivo* by the streptococcus. The buffers of the blood respond to the introduction of the acid, at first by a relatively great drop, followed by the maintenance of a constant level. The addition of more and more acid does not change the reaction of the blood, nor the CO₂ capacity. Finally, however, the buffer is exhausted and the addition of still more acid by the constantly increasing numbers of organisms brings on a state of uncompensated acidosis.

This final drop in the buffer, and the accumulation of acid resulting therefrom, causes a severe injury to the kidneys, which shows itself in the accumulation in the blood of non-protein nitrogen and creatinine. The inorganic phosphorus content also increases for the same reason, and as a result the calcium falls to abnormally low levels.

The fall in blood sugar may be accounted for by an actual direct use of glucose by the streptococcus, or indirectly by an injurious effect of the bacteria upon the liver. It is probable that both factors unite to produce the observed result. Further experiments are now in progress to determine more closely the cause of the lowering of the blood glucose.

The immediate cause of death in this condition appears to be the severe changes occurring in the kidneys, changes which result from the production of acid by the microorganisms.

SUMMARY

The following changes have been found to occur in rabbits given fatal intravenous doses of *Streptococcus hemolyticus*:

The blood sugar concentration drops at a constant rate throughout the disease, but does not reach a condition of hypoglycemia.

Glycogen is present in the liver at death.

The CO₂ capacity is lowered markedly at first, then returns to a somewhat higher level, at which it continues until the terminal stage of the disease, when the acidosis becomes very marked.

Inorganic phosphorus is markedly increased in concentration at the terminus of the disease. This increase is greater in animals showing an acute course than in those in which the disease is of the fulminating type.

Calcium also shows terminal changes, decreases occurring in both groups. In the acutely infected rabbits the decrease is less than in the fulminating group, although in both a pathological level is reached. Non-protein nitrogen and creatinine are greatly increased in the terminal stages, in both groups of animals.

It is suggested that these observations can be explained on the assumption of a large amount of acid production by the streptococcus *in vivo*.

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AN EXPERIMENTAL STUDY OF "MAD ITCH" WITH ESPECIAL REFERENCE TO ITS RELATIONSHIP TO PSEUDORABIES

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INTRODUCTION

In August of 1930 an outbreak of "mad itch" was observed in a herd of dairy cattle in Johnson County, Iowa.¹ In order to evaluate the experimental disease, an account of the clinical picture as observed in this herd will be given. The disease is so rare as to constitute a veterinary curiosity.

There were twelve animals in the herd and of these nine succumbed to the disease. No evidence of illness was observed in the remaining three. The clinical course in the nine fatal cases was rapid and in all but one an extreme pruritus was the cardinal feature of the disease. A persistent and continuous licking of an area of skin somewhere on the hind quarters, most commonly between the udder and leg, and a sudden decrease in milk production constituted the first evidence of illness. After 2 or 3 hours of continual licking the area of skin would be completely denuded of hair and bright red in color. The condition was steadily progressive and the area of abraded skin became larger. The pruritus evidently increased in intensity for the animals would rub violently against posts, barbed wire, gates, or any other convenient object, and would bite and gnaw frenziedly at themselves. By the end of 24 hours the buttocks and thighs were denuded, swollen and discolored, oozing a serosanguineous fluid, and the animals were usually down although still able to rise and walk unsteadily about. There was no temperature elevation at this time. Salivation and marked grinding of the teeth were observed in some cases. The animals became progressively weaker and died within 36 to 48 hours after the first evidence of illness. Death was preceded by clonic convulsions, violent tossing of the head, crying, and rapid shallow respiration. One

¹ Dr. Fred J. Crow of Iowa City called my attention to this outbreak of "mad itch," made the material available to me, and cooperated in the preliminary field work. I thank him heartily.

of the nine animals had failed to show any evidence of pruritus. This animal was ill for 6 days and lay listlessly in the barn lot during this period. On the last day she developed clonic convulsions, salivated, ground her teeth, and died in much the same manner as the others.

Attempts to Reproduce "Mad Itch" Experimentally

Cerebrospinal fluid in a sterile container from one cow and portions of brain from three others in 50 per cent glycerine were brought back to the laboratory on ice. A period of 2 weeks elapsed between the time of the collection of this material and its inoculation. Samples of various specimens were injected subcutaneously and intracerebrally into rabbits, guinea pigs, rats, and mice. Only one was found capable of inducing recognizable disease, this being the brain from the cow that had failed to exhibit any evidence of pruritus and had survived longer than any of the other affected animals.

Physiological salt solution suspensions of the effective brain when injected subcutaneously into rabbits reproduced the clinical picture of "mad itch" as seen in the cattle.

After incubation periods usually ranging from 50 to 75 hours the rabbits appeared uneasy and began biting and scratching mildly at the site of inoculation. There was no temperature elevation at this time. The condition became progressively worse and the biting and scratching more persistent and savage. The skin over the site of inoculation and over a large surrounding area was soon denuded of hair and abraded, and it oozed a serosanguineous fluid. As the animals became weaker, their attempts at biting and scratching were more feeble. They lay on their sides unable to rise, their respirations became rapid and in some cases noisy, and as a rule there was a marked temperature elevation. Death ensued from 6 to 24 hours after the first evidence of pruritus and it was preceded by loss of consciousness and mild clonic convulsions.

This clinical picture has been produced regularly and in quite a large series of rabbits by the subcutaneous injection of either suspensions of the original cow brain or of brains of intracerebrally infected rabbits or guinea pigs. Four out of five attempts to infect guinea pigs by subcutaneous inoculation with the original cow brain were unsuccessful and the material was also innocuous when administered subcutaneously to white rats and white mice.

Injected intracerebrally, the material produced a uniformly fatal disease in rabbits, guinea pigs, and white rats and mice.

Rabbits died in from 24 to 50 hours following inoculation and the period of actual illness was short. After an incubation period of from 20 to 40 hours the animals appeared nervous and excited and soon were observed to be blind. They became more excited and would sometimes run wildly about their cages butting their heads violently against the door. At other times they would retract their heads, lifting a front foot as though fearing attack. They frequently continued the retraction of their heads until they would rise on their hind legs and fall over backwards. No temperature elevation was to be observed until just before death when a marked rise occurred. Death was usually preceded by coma and rapid and sometimes noisy respiration. Salivation and grinding of the teeth were fairly constantly to be observed.

The above picture has been produced regularly in rabbits by the intracerebral injection of the original cow brain or by the injection of suspensions of brains from intracerebrally infected rabbits or guinea pigs. No pruritus resulted following intracerebral inoculation. The symptoms observed in guinea pigs, rats, and mice following intracerebral inoculation were similar to those seen in rabbits but they were of less violent character and the periods of incubation and survival were greater.

The disease induced in rabbits by the intranasal instillation of "mad itch" virus deserves special mention in that it differed from that induced by cerebral or subcutaneous inoculation.

For a period of 4 days following infection the animals appeared perfectly normal, ate well, and exhibited no temperature elevation. On the beginning of the 5th day the respiration of some of the animals was slightly accelerated and in one frequent sneezing was observed. The animals did not appear ill, however, and there was no temperature elevation. Death occurred suddenly on the 5th day and without warning. At autopsy the most significant pathology was found in the lungs. These were of a glistening purplish red color, heavy, and filled with a serosanguineous fluid. The bronchi and trachea usually contained an abundance of a blood-tinged frothy exudate and there were many submucous hemorrhages.

Both the clinical and the postmortem picture of the disease produced by the intranasal instillation of the virus are strikingly suggestive of a rather common bovine disease which, like "mad itch," is generally classed as hemorrhagic septicemia.

Gross Pathology

The postmortem picture of cattle dead of the spontaneous disease was always the same. The skin over the thighs and buttocks was denuded of hair, dark,

leather-like, and smeared with a serosanguineous fluid. When the skin in these regions was incised a dark red, serous, and occasionally gelatinous material was encountered in the greatly thickened subcutaneous tissue. The underlying muscle was not involved. The abdominal viscera, aside from a moderate congestion, appeared normal. The stomachs were well filled. The lungs of some of the cows exhibited areas of an edematous type of congestion; the hearts of all were spotted with numerous petechial hemorrhages, and in all cases there was an excess of pericardial fluid. Aside from moderate injection of the meningeal vessels nothing pathological in the gross was encountered in the central nervous system. There was thus no gross anatomical cause of death apparent in the spontaneous cases.

In rabbits inoculated subcutaneously, the skin in the region of the site of inoculation was denuded of hair, abraded, and moistened by a serosanguineous fluid. The underlying subcutaneous tissue was thickened, edematous, and bloody. The regional lymph nodes were enlarged and congested. The stomach, intestines, and colon were found to be well filled. The urinary bladder was, as a rule, greatly distended. The liver was strikingly of a "nutmeg" appearance. The spleen and kidneys were at the most no more than moderately congested. The heart was injected and at times spotted with petechial hemorrhages. The thymus was large, sometimes almost completely covering the heart, and was dotted with variously sized areas of hemorrhage. The lungs were a glistening purplish red, heavy, and on cut section exuded a serosanguineous fluid. The bronchi often contained a thin frothy mucus. Aside from a mild to moderate injection of the meningeal vessels nothing abnormal was encountered in the central nervous system. In contrast to the absence of gross lesions in cattle, the intensely hemorrhagic pulmonary edema could be readily held accountable for the death of experimentally infected rabbits. The gross pathological picture presented by intracerebrally inoculated rabbits was the same as that outlined for subcutaneously inoculated animals except that the lesions of the skin and subcutaneous tissue were absent. The postmortem picture presented by guinea pigs dead of experimental "mad itch" was the same as that seen in rabbits except that the lesions in the skin and subcutaneous tissue were more extreme and the pulmonary lesions less marked.

The histopathology will be reported later.

Etiology

As has been reported briefly elsewhere (1), no ordinary bacterial organism has been discovered in "mad itch" materials of proven infectivity, by methods of culture and direct microscopic examination. On the other hand, suspensions of emulsified brain of intracerebrally inoculated rabbits when passed through Chamberland L³ and Berke-

TABLE I
Filtration Experiments

Filter		Material filtered, approximately 10% suspension of rabbit brain		Time of filtration	Maximum negative pressure		Dosage of filtrate administered and route	Rabbit inoculated. No.	Result
Type	No.	Suspension	Rabbit No.		cm. of mercury	Positive pressure			
1. Seitz		A	84 and 85	min.			1 cc.		
2. "				2			0.15 ic.	95	No illness
3. "		B	76, 94, and 95	2			1 cc.	76	"
4. Berkefeld V	10	B	76, 94, and 95	2			1 cc.	114	Died—105 hrs.
5. " V	10	A	84 and 85	0.5			1 cc.	108	No illness
6. " N	6	B	76, 94, and 95	1	28		1 cc.	92	Died—96 hrs.
7. " N		A	84 and 85	0.5	48		1 cc.	106	" —76 "
8. " N	1	B	76, 94, and 95	2	37		0.15 ic.	90	" —99 "
9. " N	7	B	76, 94, and 95	2	49		1 cc.	75	" —46 "
10. " N	8	B	76, 94, and 95	2	30		1 cc.	107	" —76 "
11. " W	1	C	76, 94, and 95	2	57		1 cc.	111	" —90 "
12. " W	9	B	76, 94, and 95	3	45		1 cc.	105	" —98 "
13. " W	11	B	76, 94, and 95	2	42		1 cc.	131	" —89 "
14. " W	11	D	76, 94, and 95	6	60		1 cc.	110	No illness
15. " W	11	C	76, 94, and 95	8	60		1 cc.	109	"
16. Chamberland L ¹	11	C	110	5.5	60		0.15 ic.	109	Died—50 hrs.
17. " L ¹	1	B	76, 94, and 95	3	43		1.5 cc.	126	No illness
18. " L ¹	2	B	76, 94, and 95	5	60		1.5 cc.	125	Died—89 hrs.
	4	C	110	3.5	55		1 cc.	112	" —125 "
				10	63		1.5 cc.	113	No illness
								129	Died—101 hrs.

* sc. = subcutaneously.
ic. = intracerebrally.

feld V, N, and W filters were effective in inducing the disease in rabbits. A summary of the results is given in Table I.

In all but one of the filtrations (No. 7) infusion broth pH 7.3 was used as the vehicle in preparing the brain suspensions, but in this one case in which physiological salt solution was used no observable difference in the filtrability could be noted. In some of the experiments the brain suspensions were frozen and thawed before filtration. This procedure was unessential, for unfrozen suspensions filtered with equal facility. All suspensions were cleared of gross particles by centrifugation for 30 minutes before filtration. The moderately turbid supernatant fluid was then removed for use in the filtrations. To this was added 24 hour broth cultures of *B. prodigiosus* in the ratio of 1 cc. of culture to each 10 to 15 cc. of suspension to be filtered. 1.5 cc. amounts of the resulting filtrates were tested for sterility, as regards *B. prodigiosus*, in broth culture. All filtrates recorded in the protocol except Seitz Filtrates 2 and 3 were bacteriologically sterile. As can be seen from the table, time was an important factor in the passage of the virus through the finer pored filters. Out of five attempts at filtration through Berkefeld W filters, three filtrates in which the filtration was continued for only 2 minutes did not contain virus demonstrable in the dosage employed, while two filtrates in which the filtration was continued for 12 minutes contained quantities of virus lethal for rabbits in ordinary dosage. Difficulty was experienced in the Seitz filtrations because of the facility with which these filters passed *B. prodigiosus* in infusion broth. In one Seitz filtration in which *B. prodigiosus* was retained by the filter the "mad itch" virus was not present in demonstrable quantities in the filtrate. Two other Seitz filtrations are difficult to interpret because while both permitted the passage of *B. prodigiosus* only one permitted the passage of virus in demonstrable quantity. Animals inoculated with filtered material were adequately isolated from animals receiving unfiltered material.

The recording of the same animal in more than one experiment requires explanation. Rabbits negative in certain experiments have, after a period of observation of 2 weeks, been used over again in later experiments. The procedure may be questioned because of the possible development of some immunity following the administration of sublethal doses of virus. However, in the experiments recorded in Table I no animal, negative in an earlier experiment, failed to develop "mad itch" and die when later inoculated. This eliminates from consideration the possibility that in these experiments a confusing immunity had been set up as a result of the earlier use of the animals.

Neutralization of Virus with Immune Serum

It has been possible to induce an immunity in a guinea pig sufficient to protect that animal completely against a subcutaneous dose of rabbit brain virus always lethal for unprotected guinea pigs.

The procedure, in brief, consisted in the preliminary subcutaneous administration of the original cow brain virus which was capable of producing a fatal infection in only one out of five guinea pigs to which it was administered subcutaneously. The four guinea pigs that had been refractory to inoculation with cow brain virus were subsequently inoculated subcutaneously with rabbit brain virus known to be capable of inducing a fatal infection when administered subcutaneously to guinea pigs. Three of the animals died and the fourth (Guinea Pig 168) exhibited no evidence of illness. This fourth guinea pig has since been inoculated subcutaneously seven times with various suspensions of rabbit brain virus of proven pathogenicity for guinea pigs, and has shown no evidence of illness following any of the inoculations. The animal has been bled repeatedly from the heart at suitable intervals between inoculations with virus and the serum has been tested for its ability to neutralize virus. Typical experiments are recorded in Table II.

In the virus neutralization experiments the mixtures were usually kept at room temperature for 2 hours and at refrigerator temperature overnight before inoculation into the test animals. This preliminary period of incubation was unnecessary, for virus mixed with immune serum just before injection, into guinea pigs, was apparently as completely neutralized. Neither was it necessary to have a relatively cell-free virus suspension to attain complete neutralization, for in a number of instances the coarse uncentrifuged brain suspensions were completely neutralized by small amounts of immune serum. With the use of smaller doses of virus and larger amounts of immune serum than are recorded in Table II, it has recently been possible to attain mixtures that were completely neutral for rabbits. It has also been found that mixtures neutral for guinea pigs when administered subcutaneously are also neutral when injected intraperitoneally.

Routes of Infection

Uniformly fatal infections in rabbits have resulted following subcutaneous, intracerebral, intravenous, intratesticular, intraperitoneal, and intranasal inoculation with the "mad itch" virus. Fatal infections result from small doses of virus (0.2 cc.) injected into the tip of the ear and also from scarification through a drop of virus placed on a shaven area of skin. 0.001 cc. of the supernatant fluid of a centrifuged 10 per cent suspension of rabbit brain virus was sufficient to induce typical fatal "mad itch" in a rabbit when injected subcutaneously, whereas 0.0001 of the same virus suspension failed to infect.

TABLE II

Neutralization Tests with Immune Guinea Pig Sera

Source of virus	Amount of virus	Serum	Animal*	Result
	cc.			
Supernatant fluid of centrifuged suspension of brains of Rabbits 87, 96, and 97	1	1 cc. immune serum Guinea Pig 168	Rabbit 99	Died—122 hrs.
	1	0.5 cc. immune serum Guinea Pig 168	100	" —112 "
	1	0.1 cc. immune serum Guinea Pig 168	101	" —103 "
	1	1 cc. normal serum Guinea Pig 211	102	" — 74 " — Control
Berkefeld N filtrate of suspension of brain of Rabbit 110	1	2 cc. immune serum Guinea Pig 168	128	Died—174 hrs.
	1	0.5 cc. immune serum Guinea Pig 168	130	" —113 "
	1	1.5 cc. normal serum Guinea Pig 211	131	" — 89 " — Control
Supernatant fluid of centrifuged suspension of brain of Rabbit 140	1	1 cc. immune serum Guinea Pig 168	Guinea Pig 235	No illness
	1	0.1 cc. immune serum Guinea Pig 168	234	" "
	1	1 cc. normal serum Guinea Pig 233	236	Died— 83 hrs. Control
10% suspension of brain of Rabbit 165	1	1 cc. immune serum Guinea Pig 168	263	No illness
	1	0.1 cc. immune serum Guinea Pig 168	262	" "
	1	1 cc. normal serum Guinea Pigs 233 and 238	261	Died— 45 hrs.— Control

* All animals inoculated subcutaneously.

Guinea pigs develop fatal infections uniformly following subcutaneous, intraperitoneal, intratesticular, intracerebral, or intranasal inoculation with rabbit brain "mad itch" virus.

White rats and mice develop fatal infections irregularly following intraperitoneal inoculation, regularly following intracerebral inoculation, and are refractory to subcutaneous inoculation with the "mad itch" virus.

Distribution of Virus in the Animal Body

The "mad itch" virus tends to localize largely in the region of the site of inoculation and the lung. The brain of an animal inoculated intracerebrally will be regularly rich in virus, and the lung will also contain it. However, virus cannot be demonstrated in the heart blood, liver, and spleen. In animals inoculated subcutaneously virus is demonstrable in the bloody fluid of the subcutaneous tissue at the site of inoculation and in the lung, but it has not been demonstrable in the heart blood or spleen. Neither is it demonstrable in the brains of subcutaneously inoculated animals unless these animals have survived unusually long as the result of partial protection by immune serum. It was present in the liver of one out of three animals examined. In animals infected intranasally the virus is found in both the lung and the brain but not in the liver or blood. As dependable sources of virus the brains of intracerebrally inoculated animals or the testicles of intratesticularly inoculated animals can be relied upon. In testing for the presence of virus in various tissues 1 cc. of a 10 per cent suspension was uniformly the inoculum employed.

Animals Susceptible to the "Mad Itch" Virus

While most of our experience has been with rabbits, guinea pigs, and white rats and mice, we have tested some other species for susceptibility to the "mad itch" virus. The virus, after fourteen serial intracerebral passages in rabbits was still capable of producing a fatal infection when injected subcutaneously into its natural animal host. A calf inoculated subcutaneously with 1.5 cc. of a 6.6 per cent suspension of rabbit brain virus, after an incubation period of 4 days developed typical "mad itch" simulating in all respects the spontaneous disease as seen in the original cows in Iowa. The animal died after about 45 hours

of illness. The virus produced a fatal "itching" disease when inoculated subcutaneously into a cat. Gray field mice succumbed following intraperitoneal inoculation. Two ducks inoculated intracerebrally with the virus died after 4 and 18 days, and a suspension prepared from the brain of the first of these birds and inoculated in relatively small dosage subcutaneously into a rabbit resulted in a typical fatal "mad itch." One duck inoculated subcutaneously failed to develop any evidence of illness. The virus was innocuous for chickens when administered subcutaneously or intratracheally but following intracerebral inoculation fatal infections resulted in two out of three cases in 6 and 11 days. A suspension prepared from the brain of the first of these chickens and injected in relatively small dosage subcutaneously into a rabbit resulted in typical fatal "mad itch," proving the etiological nature of the disease to which the chickens had succumbed. Two swine inoculated subcutaneously with the virus became ill. No pruritus was observed in either animal. One animal developed what appeared to be an acute encephalitis. It lay on its side unable to rise, had clonic convulsions, and salivated. After a day of this it began to improve and could rise again although its hind quarters were weak and its gait unsteady. Recovery was finally complete. The course of the disease had been entirely afebrile. The other swine exhibited no evidence of active central nervous system involvement. It lay listlessly in its pen, did not eat, and was febrile for 6 days. Its recovery was uneventful and complete. From the clinical picture alone it would have been impossible to state definitely that the disease which developed in either hog was the result of infection with the "mad itch" virus. To obtain information on this point, blood serum from both animals after they had completely recovered was tested for virucidal properties, using as the control serum a sample of blood serum obtained from one of the swine prior to inoculation with the "mad itch" virus.

The results of this virus neutralization are given in Table III.

The virus-serum mixtures recorded in Table III were allowed to stand 2 hours at room temperature and 20 hours at refrigerator temperature before inoculation into animals. Consideration of the data indicates that, in the dilutions used, both samples of convalescent swine serum completely neutralized the "mad itch" virus whereas the

serum from one of the animals obtained before its inoculation with "mad itch" virus was devoid of virus-neutralizing properties. It therefore seems clear that the disease which developed in the swine was actually due to the injected "mad itch" virus and that swine are susceptible to the virus although more resistant than other species tested. Such a fact suggests the possible existence of a swine disease due to "mad itch" virus. It seems probable that a potent virucidal serum can be got from swine.

No definite information concerning the susceptibility of man to infection with the "mad itch" virus has been obtained during the

TABLE III
Neutralization Experiments with Convalescent Swine Sera

Virus — 10% suspension brain Rabbit 148	Serum	Guinea Pig No.*	Result
cc.			
1	0	249	Died—65½ hrs.
1	1 cc. normal serum Swine 810	248	" —70 "
1	1 " " " " 810	238	" —78 "
1	1 cc. convalescent serum Swine 810**	239	No symptoms
1	1 " " " " 810**	246	" "
1	1 " " " " 772***	237	" "
1	1 " " " " 772***	247	" "

* All animals inoculated subcutaneously.

** Serum drawn 17 days after inoculation.

*** Serum drawn 28 days after inoculation.

investigation. The writer and his helper have used ordinary precautions in handling infectious material but as autopsies have occasionally been done without gloves the bare but relatively intact skin of the hands has come in intimate contact with material of known infectivity without any untoward result. The violence of the disease, its uniformly fatal outcome, and the wide variety of species susceptible to it provide sufficient reasons for caution during work with it.

Contagiousness of Experimental "Mad Itch"

In a test of the contagiousness of experimental "mad itch" in rabbits, a doe and her litter of four were used, representing as intimate a form

of contact as was attainable under experimental conditions. All were in the same cage. The young were 22 days old at the time of their mother's infection and not completely weaned although they ate freely of the rations furnished the mother. The doe received 0.2 cc. of a 10 per cent suspension of rabbit brain virus subcutaneously in the tip of one ear and after an incubation period of 58 hours developed typical "mad itch." Pruritus and self-mutilation were limited to the inoculated ear and the side of the head. The animal died after an illness of 8 hours. No disease developed in any of the young rabbits during a period of observation of 2 weeks following their mother's death. They were all then tested for susceptibility by inoculation subcutaneously and all succumbed to the "mad itch" virus. Three other experiments have been conducted in which normal rabbits have been kept in cages with intranasally infected rabbits and in no instance has disease developed as a result of contact.

No attempt has been made as yet to determine how the disease is contracted in the field. It may be of significance that on the farm where the outbreak of "mad itch" was seen in the dairy cattle there had been a highly fatal epizootic of some sort among the rat population the preceding week. However, no rat carcasses were available for examination and no live rats could be found on the place at the time that the cattle were sick.

Viability of Virus when Glycerinated or Dried

Infectious rabbit brain stored in 50 per cent glycerol at refrigerator temperature still contains active and only slightly attenuated virus after 154 days. Similar infectious material frozen and dried by Swift's method (2) still contains active virus after 106 days' storage. Dried or glycerinated material has not been tested after longer periods of storage.

The Relationship of "Mad Itch" and Pseudorabies

The clinical picture of "mad itch" is very suggestive of pseudorabies, first described by Aujeszky (3). In fact, only after some experimentation did any doubt arise that we were dealing with the latter. White (4) in a text-book of veterinary medicine has classed "mad itch" as synonymous with pseudorabies but on no experimental basis. By

veterinary practitioners it is frequently considered a form of bovine hemorrhagic septicemia and, as such, treated unsuccessfully.

Professor Alădar Aujeszky of the Ungarische Tierärztliche Hochschule, Budapest, has kindly furnished me with a Hungarian strain of pseudorabies virus for the purpose of comparing the disease with "mad itch."

Pruritus localized to the region of the site of inoculation is the cardinal symptom after the subcutaneous inoculation of rabbits with either the "mad itch" or the pseudorabies virus. The incubation period and the period of survival following the onset of symptoms are the same with both viruses. Animals inoculated with pseudorabies virus exhibit more symptoms referable to central nervous system involvement and have a more "rabid" appearance than animals infected with "mad itch." Otherwise there are no observable differences in the clinical pictures. The findings at autopsy in an animal dead of "mad itch" are identical with those due to pseudorabies.

The distribution of virus in the body of an animal dead of "mad itch" differs widely from that in pseudorabies. Aujeszky (3), Schmiedhoffer (5), Zwick and Zeller (6), Sangiorgi (7), Carini and Maciel (8), and Isabolinsky and Patzewitsch (9) agree that the pseudorabies virus can be demonstrated not only in the local lesion but also in the brain, blood, and all highly vascular organs; it is a septicemic virus. The observation has been confirmed in this laboratory. In "mad itch," however, the virus is relatively localized, as indicated earlier in the paper.

Data concerning the filtrability of the etiological agent of pseudorabies are contradictory, and consideration of the literature would indicate that the virus is filtrable only with difficulty and irregularity at the best. Schmiedhoffer (5) found that the etiological agent was capable of passage through coarse pored filters which, however, retained *B. coli*, rotlauf, and fowl cholera bacilli. Sangiorgi (10), using a Brazilian strain of the disease, was irregularly successful in passing the virus through Berkefeld filters N and V. Aujeszky (3), Zwick and Zeller (6), Isabolinsky and Patzewitsch (9), and Bertarelli and Melli (11) were unsuccessful in filtering it. In a single filtration experiment conducted in this laboratory with pseudorabies, the virus has been found capable of passage through a Berkefeld filter of N porosity.

For this filtration brain tissue from an intracerebrally inoculated rabbit was suspended in infusion broth of pH 7.3 and frozen and thawed twice before filtration. All of the recorded attempts to filter the pseudorabies virus were carried out prior to 1914, that is to say prior to the recent advances in methods and technic in filtration.

Among the laboratory animals, rabbits are stated to be more susceptible to pseudorabies than guinea pigs, and guinea pigs in turn are more susceptible than white rats and mice (3, 5, 6). This same order of degree of susceptibility is true of "mad itch." However, Zwick and Zeller (6) found that rats were refractory to intraperitoneal inoculation with the pseudorabies virus, whereas in my experience the intraperitoneal injection of the "mad itch" virus almost always results in a fatal infection. Also chickens are considered insusceptible to infection with pseudorabies (3, 5, 6), but they succumb quite regularly to intracerebral inoculation with "mad itch" virus. Swine are stated (5) to be insusceptible to pseudorabies. They develop a non-fatal disease following inoculation with "mad itch" virus. Von Rátz (12), however, has observed pseudorabies in wild swine.

Aujeszký (3), Zwick and Zeller (6), and Schmiedhoffer (5) agree that pseudorabies is not contagious. "Mad itch" has proved not contagious even on intimate exposure, as the present paper shows.

Experimental infection with "mad itch" and pseudorabies can be induced by the same routes. However, Schmiedhoffer (5) has stated that intranasal inoculation of the pseudorabies virus proved innocuous unless the nasal mucous membrane was injured. The "mad itch" virus, on the other hand, regularly induces fatal infections when placed upon the intact mucous membrane of the nose. Using Aujeszký's strain of pseudorabies we have been successful in infecting a rabbit intranasally in the absence of any injury to the nasal mucosa.

It has been possible to obtain virucidal sera from both guinea pig and swine by the injection of the "mad itch" virus, and these sera are capable of completely neutralizing virulent "mad itch" virus as shown by the inoculation of mixtures of virus and serum into guinea pigs or rabbits. The virucidal swine serum is true convalescent serum while that from the guinea pig has been obtained without inducing any observable illness in the source animal. With a view to determining the relationship of the "mad itch" and pseudorabies viruses cross-

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neutralization tests were conducted with these sera. It was found that the sera virucidal for "mad itch" were capable of completely neutralizing the pseudorabies virus. The results of a typical experiment are given in Table IV.

There is no record in the literature of the preparation of a pseudorabies virucidal serum, and we have been unsuccessful up to now in immunizing animals.

TABLE IV
*Neutralization of Pseudorabies Virus by Anti-"Mad Itch" Sera**

Pseudorabies virus, 10% suspension of brain of Rabbit 168	Serum	Guinea Pig No.**	Result
cc.			
1	0.5 cc. normal serum Guinea Pigs 233 and 238	270	Died—81 hrs.
1	0.5 cc. anti-"mad itch" serum Guinea Pig 168	272	No illness
1	0.1 cc. anti-"mad itch" serum Guinea Pig 168	271	" "
1	1 cc. normal serum Swine 810	273	Died—93 hrs.
1	1 cc. "mad itch" convalescent serum Swine 772	274	No illness
1	1 cc. "mad itch" convalescent serum Swine 810	275	" "

* The virus-serum mixtures were allowed to stand 2 hours at room temperature and 14 hours at refrigerator temperature prior to inoculation into animals.

** All animals inoculated subcutaneously.

From the data presented in this paper the writer is led to the tentative conclusion that the virus of "mad itch" and that of pseudorabies are the same; the differences observed may be accounted for on the basis of slight differences in strains of the same virus. Further information as to their relationship must await completion of cross-neutralization tests with "mad itch" virus and pseudorabies virucidal serum.

SUMMARY AND CONCLUSIONS

The clinical picture and gross pathology of spontaneous and experimental "mad itch" have been described and the inciting agent has been

shown to be a filtrable virus. It has been possible to prepare virucidal serum capable of neutralizing the virus. Fatal infections are regularly produced in rabbits when the virus is administered subcutaneously, intracerebrally, intravenously, intratesticularly, intraperitoneally, intranasally, or when it is dropped on a scarified area of skin. Its infectivity for other species by various routes is reported upon. The rabbit, guinea pig, white rat, white mouse, gray field mouse, cow, cat, duck, chicken, and hog are susceptible to experimental infection. The disease is not contagious under laboratory conditions and the virus is restricted in the animal body largely to the region of inoculation and the lung. The virus can be stored for relatively long periods in 50 per cent glycerol or in the dried state.

A comparison of "mad itch" with pseudorabies leads to the tentative conclusion that the inciting agents of both are the same, although the strains of the two viruses that are under study possess readily demonstrable differences

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LOCAL ORGAN HYPERSENSITIVENESS

III. FURTHER OBSERVATIONS ON ITS EXPERIMENTAL PRODUCTION IN THE RABBIT EYE

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PLATE 24

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Research in the field of hypersensitivity has been largely concerned with the reactions of animals which have been rendered *generally* sensitive by the parenteral introduction of a foreign protein. Certain experimental work, however, and a considerable amount of clinical experience has indicated that, under appropriate conditions, *local* areas of an animal body may become acutely sensitive to contact with a foreign protein while the remainder of the animal participates little or not at all in this local reaction. An early observation in this regard was that of Mackenzie and Hanger (1) who demonstrated that areas of skin previously injected with living streptococci became red and swollen if the same material was reinjected into other skin areas at subsequent periods.

The purpose of this work was the further investigation of the possibility of actively sensitizing local portions of the animal body.

From a review of previous attempts to produce local hypersensitivity it seemed that the critical requirement was the maintenance of a set of experimental conditions which would keep antigen in juxtaposition with body cells for a relatively long period of time. The structure of the anterior chamber of the eye presents a feature which probably allows this condition to be fulfilled. We had observed that heterologous erythrocytes when injected into the anterior chamber of the rabbit's eye would persist for several days. On this account the anterior chamber of the eye was the initial area chosen to test for

the production of local hypersensitivity. The experiments reported here have shown that the anterior chamber of the rabbit eye is one of the sites in which local organ hypersensitiveness can be produced.

Method

The experimental method which has been briefly described in a previous communication (2) was as follows:

The animals used were for the most part albino rabbits. The albino rabbit was preferable because hyperemia of the iris and slight changes in the opacity of the anterior chamber fluid or cornea were more readily detected. For antigen a large number of proteins were employed, either singly or in mixtures containing as many as ten substances. These antigens may be divided into two groups. One group consisted of non-noxious, relatively indifferent agents such as the aged serum or red blood cells of other species of animals, egg albumen, and casein. The second group consisted of bacterial bodies, their fractions, and their products. In this paper, with one exception, only the experiments carried out with non-noxious agents, such as foreign sera, will be described. The sensitization of an animal was accomplished by the preparation of one anterior chamber only, except where a special experiment called for the use of both eyes. This left the unprepared eye as a control. To inject the anterior chamber with a sensitizing dose of antigen, 4 per cent cocaine was first dropped into the conjunctival sac. The animal was then held by an assistant and approximately 0.25 cc. of anterior chamber fluid removed. This was accomplished by inserting a 27 gage hypodermic needle, with tuberculin syringe attached, tangentially into the anterior chamber at the limbus.* Without removing the needle the syringe was exchanged for one containing the foreign protein. Quantities of foreign protein varying from 0.1 to 0.25 cc. were injected into the anterior chamber through the same needle. The actual quantity of any one antigen so injected depended on the number of antigens which were mixed together to form the sensitizing dose. For example, in one experiment 0.15 cc. of a 40 per cent saline suspension of fresh egg white was used, which represented 0.06 cc. of undiluted egg white. In another experiment, 0.2 cc. of a mixture of foreign proteins consisting of citrated guinea pig, sheep, and pigeon blood, horse serum, 1 per cent casein, 5 per cent egg white, and an anhemolytic streptococcus vaccine was employed. Considering each blood as composed of two antigens, serum and red cells, this made a total of ten different antigens and a total volume of 0.02 cc. or less for each individual antigen.

The replacement of anterior chamber fluid by such foreign substances caused an immediate sterile inflammation of the iris and conjunctiva which persisted from 2 days to a month and varied in intensity, depending on the quantity and

* We are indebted to Dr. Lloyd Morgan of the Ophthalmology Department at Presbyterian Hospital who demonstrated the method to us.

nature of the foreign material and the individual peculiarity of the animal. The mildest type of reaction consisted only of a slight injection of the vessels of the conjunctiva, sclera, and iris, and a slight clouding of the anterior chamber. A severe reaction consisted of intense hyperemia of the conjunctiva with marked chemosis and lacrimation, a clouding of the anterior chamber fluid and cornea, sometimes sufficient to obscure the pupil completely, and an extension of the sclera down over the cornea. Even with a very severe reaction the eye may return to practically a normal appearance in the course of several weeks.

To test for the sensitivity of the prepared eye, 1 cc. of the antigen or of one ingredient of the mixed antigen used to sensitize the eye, was injected intravenously. This intravenous shocking injection was given only after the initial reaction in the eye, induced by the foreign substance, had disappeared and there was no further injection of the vessels. About 1 hour after the intravenous injection of the antigen, hyperemia of the iris, conjunctiva, and sclera, chemosis and lacrimation began to appear. The anterior chamber in severe reactions became slightly cloudy and corneal vessels reappeared. This reaction reached its height in about 5 hours and faded in about 24 hours, although a slight hyperemia sometimes persisted for 48 hours or longer.

Incubation Period of the Local Hypersensitiveness

Eyes which had been sensitized with a foreign protein uniformly failed to respond to the intravenous injection of the homologous substance until at least the 5th day following the introduction of the antigen into the anterior chamber. Although slight inflammatory responses could be produced in a very few animals on the 6th to the 10th day after sensitization, it was quite evident from the majority of experiments that the eye response reached a maximum from the 10th to the 15th day or even later after local sensitization. The technical difficulties incident to the evaluation of this point centered around the fact that the major portion of the inflammation produced by the injection of antigen into the anterior chamber usually did not subside before at least the 3rd to the 5th day. By a careful selection of animals and of antigen this was overcome. The fact that the incubation period appears to be about 2 weeks would seem to place this reaction in the group usually considered as due to an antigen-antibody complex.

The Specificity of the Vascular Response in the Sensitized Rabbit Eye

In a previous paper (2) an experiment was described which illustrated the specificity of the local eye reaction. Briefly stated, two rabbits were prepared by the injection of 0.15 cc. of a 40 per cent saline

suspension of guinea pig red blood cells into the right anterior chamber, two received 0.15 cc. of a 40 per cent saline solution of fresh egg white and one animal was similarly prepared with physiological saline solution. 13 days later the intravenous injection of guinea pig red blood cells or fresh egg white produced an eye reaction in only those animals injected in the eye with the homologous antigen. Fig. 1 illustrates the reaction.

We have arbitrarily recorded the reactions as of 1, 2, 3, or 4 plus intensity depending upon the amount of hyperemia which develops in the vessels of the conjunctiva, sclera, and iris. Thus the inflamed eye B recorded in Fig. 1 shows what we term a 2 plus reaction whereas eye D shows a 3 plus reaction. In describing the reactions hereafter they will be referred to as exhibiting 1, 2, 3, or 4 plus intensity, or in the case of mild hyperemia they may be spoken of as $\frac{1}{4}$ or $\frac{1}{2}$ plus reactions.

The results of the experiments just described appear to show that the reaction is a specific one and not dependent upon preliminary injury to the eye. Further evidence in this regard is that adduced from observations in which it was found that anterior chambers in which an initial tissue injury had been produced with typhoid vaccine would not respond subsequently when the various proteins used for sensitization in the eyes of the other animals were injected intravenously.

Another experiment which demonstrated that non-specific injury to an eye would not cause it to become inflamed upon intravenous injection of a foreign protein was carried out with iodine, albolene, saline, and glycerine. Each of these substances, separately, was injected into the right eye of four series of three rabbits each. During the 3rd week after this preliminary injection, when the eyes had returned to a non-inflammatory condition, 1 cc. of 20 per cent fresh egg white, and 5 days later, 0.8 cc. of a 33 per cent suspension of guinea pig red blood cells were injected intravenously into these twelve animals. These injections failed to produce any hyperemia or other discernible reaction in the eyes which had been prepared with the non-antigenic irritating substances.

Throughout most of the experiments in which at least 1000 eye reactions have been observed, the contralateral eye has acted as a

control for the reacting sensitized eye. In nearly all instances the control eye has failed to react in the presence of the opposite reacting eye. However, in a small number of instances the non-sensitized eye has shown very slight vascular response, particularly when the sensitized eye was exhibiting a marked inflammatory response. The infrequency of this result and the slight nature of the response in the normal eye failed to cause any difficulty in the evaluation of the phenomenon. The significance of the slight reaction in the contralateral eye is not as yet clear.

Wessely (3) found that after the injection of horse serum into the rabbit cornea a local reaction appeared which subsided in 48 hours. 2 weeks later a spontaneous relighting of the same cornea appeared. In our experiments we have noted the spontaneous inflammation of the eye only in rare instances. It is very transient, of slight degree, and apparently does not recur significantly thereafter.

Types of Desensitization

Repeated intravenous injections of the same antigen resulted in a much decreased to absent local eye reaction. We interpreted this failure of response as a desensitization phenomenon. Usually desensitization did not prove to be permanent, although in one animal massive injections of antigen did finally suppress the eye reaction. The two following protocols illustrate types of desensitization and the first is typical of several similar observations.

Rabbit 103 on Dec. 30, 1929, received 0.1 cc. of a 30 per cent suspension of fresh egg white into the left anterior chamber. On Jan. 2 and every day thereafter through Jan. 10, the animal received 1 cc. of a 30 per cent suspension of fresh egg white intravenously. The first three injections on Jan. 2, 3, and 4, produced no reaction in the eye; but the injections on Jan. 5 and 6, 6 and 7 days respectively after the animal's initial sensitizing injection, produced just barely perceptible hyperemia, while the injection given on Jan. 7, the 8th day after sensitization, provoked a 1 plus injection with 1 plus edema and lacrimation in 1½ hours. The following day there was only ½ plus response to the intravenous injection of egg white and on the 10th of January, the injection produced no response. The rabbit's eye had, therefore, been able to respond with hyperemia, edema, and lacrimation of a moderate degree, following intravenous injection of the antigen, despite the fact that it had received three daily injections of the same antigen intravenously in the incubation period of sensitivity. But subsequent injections were again without appreciable effect. 17 days later another similar intravenous

injection elicited a $\frac{3}{4}$ plus reaction. In another 17 days another intravenous injection produced only a $\frac{1}{2}$ plus reaction. 5 days later, on Feb. 18, 5 cc. of the egg white suspension was given subcutaneously and produced, as might have been expected, an Arthus phenomenon in the skin, but no eye response. About 3 weeks later, on Mar. 13 and 17, the animal was given 5 and 7.5 cc., respectively, of the egg white intraperitoneally with $\frac{1}{4}$ plus reaction on the 1st day and $\frac{1}{8}$ plus reaction on the 2nd day. The animal was now allowed to rest for over 4 months and was then retested with an intravenous injection of 2 cc. of a 25 per cent suspension of fresh egg white. The eye responded with a $\frac{3}{8}$ plus reaction and slight amount of edema.

It appears, therefore, that the degree of sensitivity produced in the rabbit's eye can be diminished by repeated injection of this antigen. With overwhelming doses of the antigen it can apparently be completely suppressed, as the following protocol illustrates.

Rabbit 102 (Rabbit B of Fig. 1) received 0.15 cc. of guinea pig red blood cells into the right anterior chamber on Dec. 13, 1929. This animal responded with a 2 plus injection, edema, and lacrimation on the 26th of December, 5 hours after the intravenous injection of 1 cc. of a 40 per cent suspension of the homologous red cells. From Dec. 28 through Jan. 10, the animal received similar daily intravenous injections. The response decreased to only $\frac{1}{2}$ plus on the 31st, but on Jan. 1st was again 2 plus. This sudden reacquisition of sensitivity slowly decreased again until on Jan. 9 there was no further response to the antigen. In February and March the animal was given multiple injections of guinea pig red blood cells amounting to a total of 44 cc. of a 10 per cent suspension in an attempt to produce a high titered lytic serum for guinea pig cells. The sensitized eye did not react at any time during this course of treatment. $4\frac{1}{2}$ months later an intravenous injection of 2 cc. of 30 per cent cells failed to produce any effect in the sensitized eye.

This desensitization is not due to the long interval of time which elapsed between sensitization and the final testing for reactivity of the eye, for many animals which have received only a few intravenous injections of the antigen are still sensitive after 8 months.

If adrenalin is administered intravenously when the local eye reaction is at its height an almost immediate and characteristic effect is noted. In three animals in which the reacting eyes exhibited conjunctival injection, slight chemosis, and lacrimation, the intravenous injection of 0.2 cc. of adrenalin 1:1000, was followed by an almost immediate blanching of the inflamed conjunctiva and a progressive diminution of the edema and lacrimation. In two of the animals a

partial return of the inflamed state $\frac{1}{2}$ hour after the injection of the adrenalin was again controlled by a repetition of the dose. That this reaction to adrenalin is not specific for a hypersensitive inflammation is shown by the fact that eyes showing conjunctival injection, chemosis, and lacrimation secondary to chemical injury also show the same response to the parenteral injection of the adrenalin.

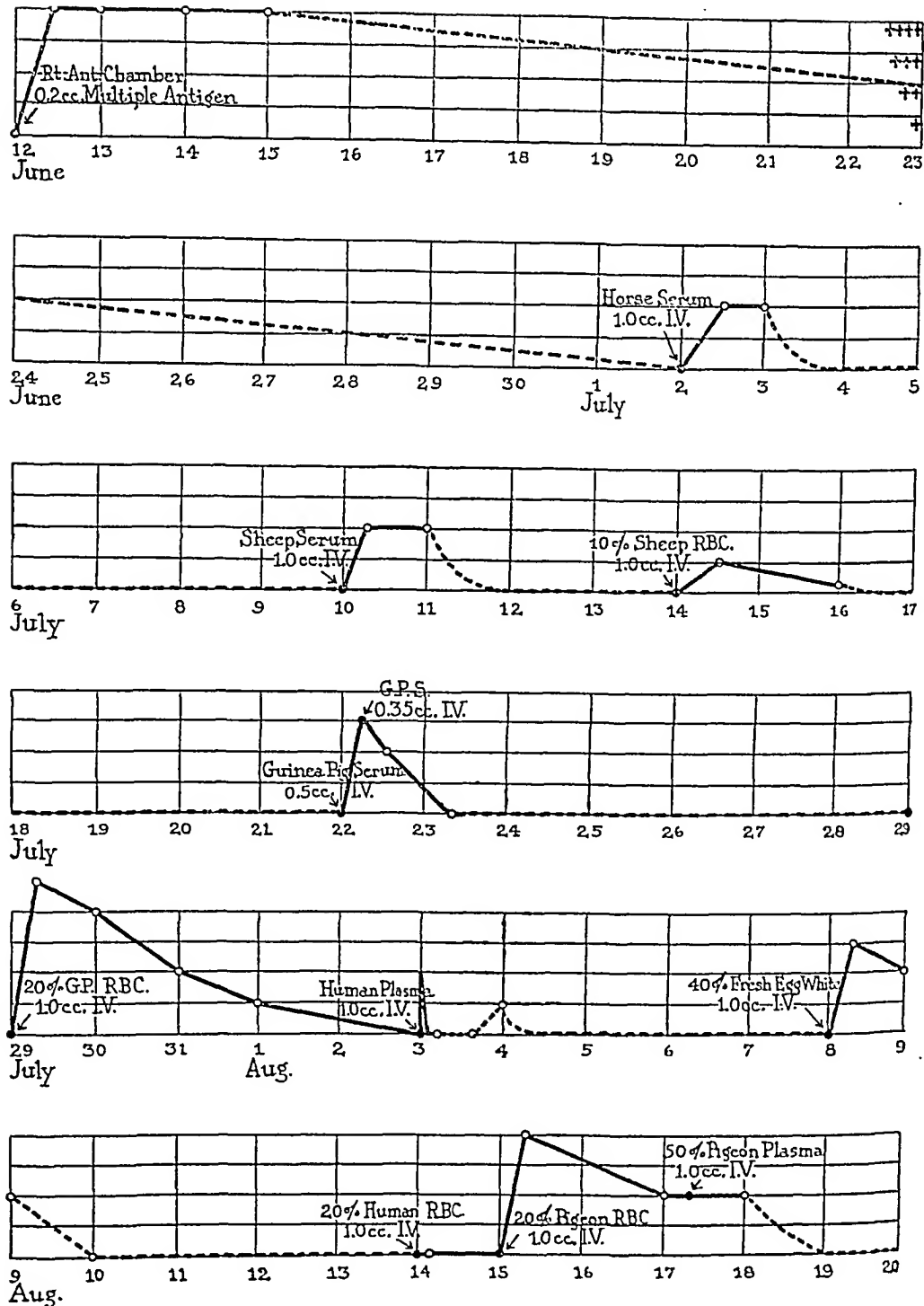
Repeated Vascular Response in the Sensitized Rabbit Eye

In order to avoid the phenomenon of desensitization and to work with an eye which might be kept more or less constantly in a state of sterile inflammation, a "multiple" antigen was used to sensitize the eyes of a new series of rabbits. This was prepared by mixing together the citrated or defibrinated blood of a number of animals along with some other foreign proteins.

The first multiple antigen consisted of citrated guinea pig, sheep, and pigeon blood, horse serum, 1 per cent casein, 5 per cent egg white, and an anhemolytic streptococcus vaccine. Considering each blood as composed of two antigens, serum and red cells, this constituted a total of ten different antigens. One anterior chamber, or in some cases both anterior chambers, were injected with 0.2 cc. of this mixture after the removal of 0.2 cc. of anterior chamber fluid, as previously described. The local sensitiveness of the eyes was then tested by the separate intravenous injection of each ingredient of the multiple antigen, after the initial reaction in the eye, due to the presence of the foreign proteins, had subsided. The sensitized eye responded to each succeeding injection by a sterile inflammatory reaction already described. The time allowed to elapse between the intravenous injections of each of the separate antigens varied from 1 to several days.

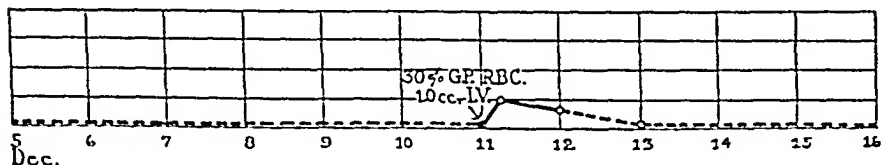
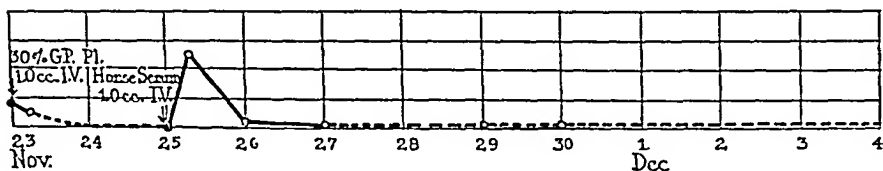
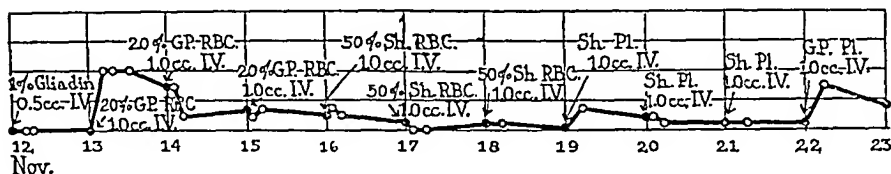
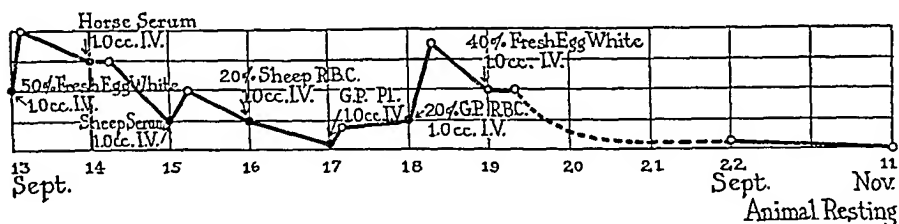
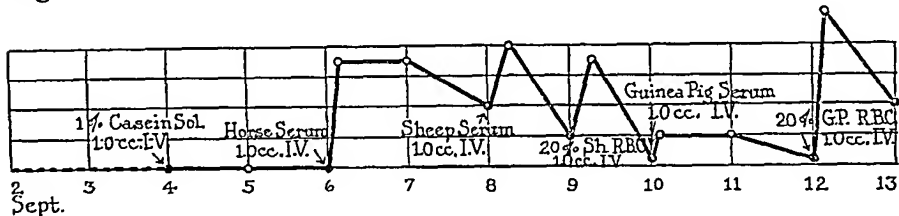
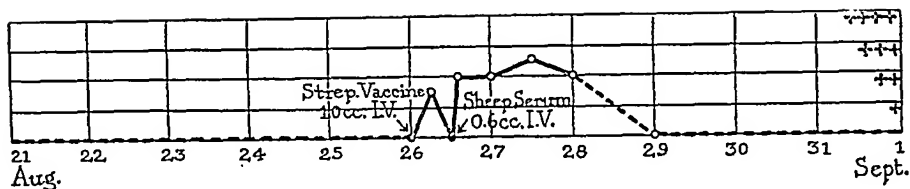
It was found that the eye reaction which had been produced previously with the use of a single antigen could now be produced by the intravenous injection of each of the antigens which had been used to sensitize the eye. It became possible by the daily injection of a different antigen to maintain a rabbit's eye in a state of sterile inflammation for a period of a week or more. A typical protocol illustrates the means by which the eye was kept continually inflamed and will bring out certain points concerning desensitization phenomena in the eyes in which the multiple antigen was utilized.

In Graph 1 the history of Rabbit 22-91 is followed from June 12 to Dec. 16, 1929. On June 12 this animal was prepared by an injection into the anterior chamber of the right eye of 0.2 cc. of a multiple antigen consisting of horse serum,



GRAPH 1. Rabbit 22-91. Prepared June 12, 1929, by the injection of 0.2 cc. of multiple antigen into the anterior chamber of the right eye. The multiple antigen consisted of horse serum, guinea pig blood, sheep blood, pigeon blood, an anhemolytic streptococcus vaccine, 1 per cent casein solution, and fresh egg white.

The inflammatory reactions in the eye incident to the initial injection into the eye and the subsequent intravenous injection of fractions of the antigen are



diagrammatically represented as of 1 plus (+), 2 plus (++), 3 plus (+++), or 4 plus (++++) intensity. Points of note on the graph are: (1) the eye response to a single intravenous injection of antigen; (2) the sustained nature of the inflammatory reaction following the daily injection of different fractions of the multiple antigen; (3) the diminished to absent response following the repeated daily injection of the same antigen; and (4) the return of sensitivity after a long rest period.

guinea pig, sheep, and pigeon blood, an anhemolytic streptococcus vaccine, 1 per cent casein, and fresh egg white. A few hours after the injection the eye was intensely hyperemic and there was some edema of the conjunctiva and lacrimation. The hyperemia is arbitrarily designated upon the graph as of 1 plus, 2 plus, 3 plus, or 4 plus intensity. This initial inflammation completely subsided so that on the 1st of July the eye appeared practically normal. On July 2 it can be seen from the graph that an intravenous injection of 1 cc. of horse serum produced a hyperemia of 2 plus intensity.

From the graph it will be seen that the sensitized eye reacted to the intravenous injection of all the ingredients of the multiple sensitizing antigen except casein.

Although specificity had already been proved for an animal sensitized with a single antigen, to control the specificity of the reaction in this new experiment, human plasma, human red blood cells, and gliadin, substances not in the sensitizing multiple antigen, were used. Following the intravenous injection of human plasma, the sensitized eye showed a momentary blush which faded within a half-hour. Two subsequent readings that day were negative. There was an unexplained 1 plus injection of the eye the next day. This is one of two animals of the series of ten animals similarly injected at this time which showed a reaction to human plasma. Human red blood cells and gliadin injected intravenously provoked no reaction in the sensitized eye.

On Sept. 6, about 3 months after sensitization, the animal was injected intravenously on successive days with the various constituents of the multiple antigen. It may be noted that on the whole the eye responded with more marked hyperemia now, 3 months after sensitization, than previously. From Sept. 6 to 14 there was a continuous inflammation except for 1 day's rest. On Sept. 14 the intravenous injection of the same antigens was repeated again. The inflammation produced by this rapid repetition of the exciting stimulus was less intense than during the previous week. After a rest of over 3 weeks this desensitization process was carried further by the repeated daily injections of a single ingredient of the antigen, guinea pig red blood cells, until no further response in the eye was elicited. After another rest of 4 weeks, however, intravenous injection of guinea pig red blood cells again produced a mild hyperemia.

Duration of State of Sensitivity

If animals with sensitized eyes are not utilized for 2 months, intravenous injection of the homologous antigen at that time produces a typical local eye response. It is reasonable to suppose that the interval between the sensitizing and the initial shocking date may be much longer. This can be inferred not only from the fact recorded above, but also from the fact that an animal which has been sensitized with a multiple antigen and then shocked many times by the repeated intravenous injection of the factors of that antigen will still give an

eye reaction at least 8 months after sensitization. These reactions obtained many months after sensitization by repeated intravenous injections are, with few exceptions, not as severe as the original reactions. However, as already pointed out, permanent, complete desensitization has occurred only under those conditions in which massive doses of antigen have been injected.

Variability of Eye Reactions in Different Rabbits

If the experimental animals were in good health and had been adequately fed and watered, it was rare to find a rabbit which failed to react, at least to a slight degree, to some of the shocking antigens. Although a completely negative reactor was only occasionally encountered in a series of about 200 rabbits, it was found that reactivity diminished or disappeared if the animals were suffering from ear canker, diarrhea, snuffles, or skin disease.

From a survey of the degrees of inflammatory response in the actively sensitized eyes it is apparent that the rabbits may be divided into three groups. A small group consists of animals who are excellent reactors. This group maintains its sensitivity for many months and resists desensitization measures. A second group which makes up the majority of the series, consists of moderate reactors whose response may vary over rather wide limits and whose ability to react becomes spontaneously diminished over a period of months. It is in this group, particularly, that one observes that eyes which have been sensitized to the multiple antigen exhibit a different response to the various ingredients of the antigen when given intravenously. This variation of response to different antigens is not a constant feature nor can it be evaluated from the present series. Some animals may react better to guinea pig red blood cells than to guinea pig plasma early in their experimental life, only to have this condition reversed several months later. The extent of the variation of this phenomenon allows no generalizations.

The final group which consists of about one-fourth of the series is made up of animals in which only a minimal sensitivity is demonstrable. Irrespective of the antigens used, the spacing of the shocking intervals and other modifications of the experiment, these rabbit eyes, though reacting repeatedly, manifest a $\frac{1}{2}$ plus to a 1 plus reaction,

whereas the good reactors will be found to have a 3 to a 4 plus reaction. Furthermore, when these poor reactors are resensitized in their opposite normal eyes and the shocking regime carried out anew, the same diminished reactivity is still apparent.

Effectiveness of Subcutaneous Injections of Antigen in the Production of the Eye Response

Although most of the attempts to demonstrate local eye sensitivity depended upon the intravenous shocking route, it was found that the subcutaneous injection of the various antigens produced comparable results. No attempt was made in this work to titrate the minimal effective dose when the subcutaneous route was utilized. For the most part, five to ten times the intravenous dose of 1 cc. was administered. There seemed to be no striking lag in the eye response after subcutaneous as opposed to intravenous injection. In a few of the animals a typical Arthus type of inflammation appeared at the site of the subcutaneous injection. In the animal in which the most striking Arthus reaction took place there was practically no eye response. This observation was of interest since none of the animals in which the marked eye reactions were produced by subcutaneous injections showed a concomitantly significant Arthus phenomenon.

DISCUSSION

The accumulated data would seem to prove that a locally injected area of the body such as the anterior chamber of the rabbit eye may manifest sensitivity following the parenteral injection of homologous antigen. Most of the previous studies on ocular sensitivity have dealt with local eye reactions in a generally sensitive organism. Such work has led, particularly, to the clinical use of conjunctival tests for general sensitivity to such substances as horse serum and the products of the tubercle bacillus.

The bibliography on this problem is noted in Reference 2. A number of workers in ophthalmology have attempted to explain certain types of conjunctivitis, sympathetic ophthalmia, and keratitis on the basis of "ocular anaphylaxis." Schieck, Lemoine, Tooker, and Pasteur Vallery-Radot among others have contributed clinical observations in this regard. Kodama found that in sensitized guinea pigs the introduction of the shocking agent "into the orbit" produced

stimulation of plain muscle, circulatory disturbances, and hypersecretion of eye glands. In attempting to explain the manifestations of sympathetic ophthalmia on the basis of "anaphylactic phenomena," Elschnig, Kummel, Wissman, Fuchs and Meller, von Szily, and Woods have made certain contributions regarding the question of whether the injured uveal tract of one eye may act as a sensitizing and subsequent shocking antigen to the other eye. Stanculeanu and Nita have demonstrated the Arthus phenomenon in the conjunctiva by the use of horse serum, and Kirchner has sensitized the cornea of the rabbit to *S. scarlatinac* toxin, demonstrating sensitization by repeated injections into the cornea. Schoenberg injected human serum into the anterior chamber of two rabbits and tuberculin into the anterior chamber of two other rabbits. Intravenous injection of the homologous antigen 2 weeks later produced no significant eye reaction. Brown and Dummer injected increasing doses of hemolytic streptococcus vaccine into the conjunctiva of two rabbits for 4 days and then injected a suspension of the living organisms intravenously on the following day. No eye reactions were noted.

Attempts to reactivate a locally sensitized eye by parenteral injection of the homologous antigens have been few. Von Szily (4), using sheep lens as an antigen, injected the vitreous humor of the rabbit eye and several weeks later injected the same antigen intravenously. He obtained an inflammation of the prepared eye appearing in about 6 hours and persisting for 48 hours or longer. Woods (5) injected uveal pigment into the vitreous humor of one eye in dogs. In two of these animals subsequent intraperitoneal injection of the antigen produced bilateral eye reaction. Riehm (6) injected horse serum about the iris of one eye in the rabbit and found that subsequent intravenous injections of the antigen gave him a bilateral reaction. Mott and Kesten (7), using essentially the same technique that we employed, were able to reactivate a rabbit's eye sensitized to *Monilia psilosis* by injecting a polysaccharide fraction of this organism intravenously 2 weeks to 4 months after the initial sensitizing injection.

The significance of the production of local areas of actively sensitized tissue is threefold. It apparently offers a logical experimental basis for the explanation of certain types of ocular inflammation. Suffice it to mention the relighting of a tuberculous keratitis following the subcutaneous injection of tuberculin. The method offers furthermore an opportunity for the study of delicate antigenic relationships. Finally the obvious implication is that under appropriate experimental conditions similar local areas of actively sensitized tissue may be produced in other organs.

CONCLUSIONS

1. Rabbit eyes sensitized with guinea pig red blood cells or fresh egg white respond with an inflammatory reaction following the intravenous injection of the homologous antigen, but not the heterologous.

2. Two-tenths of 1.0 cc. of a multiple antigen containing ten separate ingredients, or in other words, 0.02 cc. of each foreign protein, when introduced into the rabbit's anterior chamber, is sufficient to produce an altered ocular reactivity such that when 1 cc. of one of the ten antigens is introduced intravenously the eye shows hyperemia of the iris and conjunctiva with more or less edema and lacrimation during the next 24 hours.

3. For as long as 8 months after sensitization eyes will respond with an inflammatory reaction following the intravenous injection of fractions of the total antigen.

4. Repeated daily intravenous injections of a single antigen usually produce no reaction in the sensitized eye after the first few days. Injection of different antigens intravenously on succeeding days produces a continued sterile inflammatory process in the sensitized eye. After the total number of single antigens has been injected, repetition of these injections now fails to produce a similar response. Instead, the eye reaction is at a much lower level and the inflammatory response is manifested only to a few of the antigens injected intravenously.

5. Unless massive doses of antigen are used to desensitize, permanent desensitization of the eye has not occurred in animals which have been followed for at least 8 months. Animals may develop maximal eye responses following repeated intravenous injections of the same antigen, if sufficient time has elapsed between injections. Nevertheless, the eye reactions which can be elicited 6 or 7 months after sensitization are less intense than the initial responses.

6. The ability of the eye inflammation to light up following the intravenous injection of homologous antigen is not due to an initial tissue injury as proven by the fact that the reaction is specific and anterior chambers injured with typhoid vaccine, iodine, saline, glycerine, or albolene will not respond subsequently when the various proteins used for sensitization of the other eyes are injected intravenously.

7. It has been impossible to demonstrate sensitivity in the eye by the intravenous shocking route until at least the 5th day following introduction of the antigen into the anterior chamber.

8. The eye reaction can be produced by the subcutaneous as well as the intravenous injection of antigen.

9. Rabbits vary considerably in the intensity of the eye reaction which can be elicited in them, but only rarely was an animal found which failed completely to give a reaction.

We wish to thank Dr. F. P. Gay and Dr. A. R. Dochez for their many helpful suggestions during the course of this work.

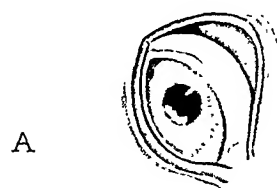
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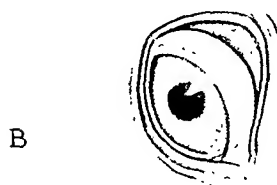
EXPLANATION OF PLATE 24

FIG. 1. Reactivation of locally sensitized eyes.

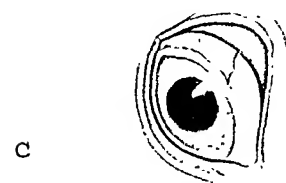
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after introduction into
anterior chamber of :



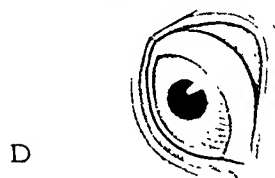
Guinea pig red blood cells



Guinea pig red blood cells



Egg albumen

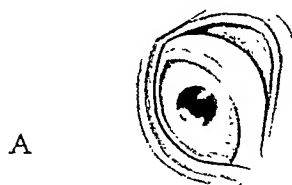


Egg albumen

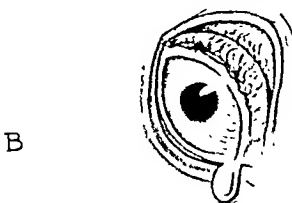


Physiological saline solution

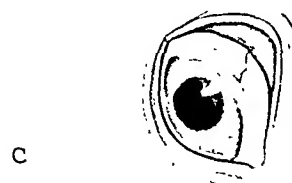
Same day-eyes 5 hours
after intravenous in-
jection of :



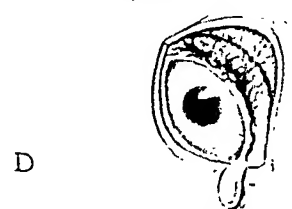
Egg albumen



Guinea pig red blood cells



Guinea pig red blood cells



Egg albumen



Guinea pig red blood cells
(24 hrs. later) Egg albumen

LOCAL ORGAN HYPERSENSITIVENESS

IV. INFLAMMATION PRODUCED IN THE ACTIVELY SENSITIZED RABBIT EYE BY THE INTRODUCTION OF HOMOLOGOUS ANTIGEN INTO THE GASTROINTESTINAL TRACT

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(Received for publication, April 16, 1931)

The demonstration that an eye which has been actively sensitized locally becomes acutely inflamed following the intravenous or subcutaneous injection of homologous antigen (1, 2) suggested the possibility that the introduction of the same antigens into the gastrointestinal tract might produce similar effects. There was evidence from the work of others that the normal human and guinea pig gastrointestinal mucosa is not an effective barrier to the passage of small amounts of unchanged protein into the blood stream.

Walzer (3) has reviewed the literature on this subject through 1927. The following has been found.

1. Prolonged feeding of egg to rabbits results in the appearance of specific precipitins in their blood (Uhlenhuth).

2. Specific precipitinogen appeared in the blood and urine of normal human beings about 2 hours after the ingestion of egg (Ascoli; Croftan). However, Hamburger and Sperk failed to demonstrate unaltered antigen in the circulation and urine of normal individuals after the ingestion of "physiological" amounts of protein.

3. Specific precipitins appeared in the blood of animals and normal adult humans only if massive doses of protein were fed or if the intestinal mucosa was traumatized (Ganghofner and Langer). In the newborn, however, "the enteral canal was permeable to *physiological* amounts of ingested protein" (Ganghofner and Langer; Anderson, Schloss, and Myers).

4. The enteral absorption of antigenic protein by normal or marasmic infants led to (a) the appearance of specific precipitins in the blood, and (b) specific cutaneous

hypersensitiveness. These two phenomena were related (Du Bois, Schloss, and Anderson).

These specific precipitins appeared very promptly, were of slight degree, and disappeared quickly in the normal infants, whereas in marantic infants the precipitin formation tended to be retarded, reached a higher level, and persisted for a longer time than in the normal infants (Anderson, Schloss, and Myers).

5. "The blood serum and urine of animals collected after the enteral administration of large amounts of protein, contained enough undigested protein to induce, by its injection, sensitivity in other animals" (Van Alstyne and Grant; Van Alstyne).

Guinea pigs who had been fed horse serum or horse meat would suffer anaphylactic shock when they were subsequently injected intraperitoneally with horse serum (Rosenau and Anderson).

Similar sensitization could be accomplished when egg white was administered orally or rectally (Maie). Maie was unable to produce anaphylactic shock in such animals by a second *enteral* injection of egg white. However, guinea pigs sensitized intraperitoneally with horse serum develop anaphylactic shock following the enteral administration of the homologous antigen (Hettwer and Kriz).

The most pertinent literature, however, concerns the experiments of Prausnitz and Kuestner (4) and Walzer (3). The former workers made an observation on passive local sensitization which has been repeatedly confirmed by others. They showed that the injection of the serum of a patient hypersensitive to fish into the skin of a normal individual conferred specific sensitivity in that site. This was proven by the production of a wheal and erythema reaction when the homologous antigen (fish extract) was introduced into the injected area.

Walzer used this principle in an ingenious experiment in which he demonstrated that the skin of 44 out of 50 normal individuals, in whom the intradermal passive transfer of hypersensitive serum had been made, developed a typical wheal and erythema at the site of injection in 2 to 75 minutes after the ingestion of the homologous protein. Walzer concluded that "the local reaction was due to the union of egg reagins, at the sensitized site, with circulating egg protein which had been absorbed, in an unaltered form, from the gastro-enteral canal."

The purpose of our experiments was to show that the similar passage of unaltered protein from the gastrointestinal tract into the blood stream of rabbits would be effective in relighting an *actively* sensitized local area of the body such as the anterior chamber of the eye.

Method

Rabbit eyes were sensitized under cocaine anesthesia with either single or multiple antigens by the method described in a previous paper. A series of 38 rabbits was selected with past histories which demonstrated their particular degree of eye response to the intravenous or subcutaneous injections of antigen. Some were uniformly poor reactors, whereas others of the group had previously shown marked

ocular inflammation following the intravenous or subcutaneous shocking dose of antigen. By means of a small stomach tube individual antigens such as 33 per cent fresh egg white, whole guinea pig plasma, whole sheep serum, or 10 to 33 per cent guinea pig, human, or cat erythrocytes in isotonic or hypotonic salt solution were introduced into the stomach. The volume of fluid introduced varied from 20 to 40 cc. No attempt was made to carry out these studies on rabbits which had been fasted over long periods, although one-third of the animals were deprived of food for at least 48 hours prior to the gastric introduction of the shocking antigen. This was apparently not a sufficient fast period for these animals since the stomach of one of them fasted for 2 days still contained a large amount of partially digested carrots.

The state of both the experimental and control eyes was observed prior to the gastric introduction of the antigen, as well as frequently thereafter.

DATA AND DISCUSSION

Of 38 such experiments on 37 rabbits weighing from 2 to 3 kilos, there were eleven instances, or 29 per cent, in which a characteristic eye response followed the introduction of the homologous antigen by stomach tube. Minor eye reactions which were probably of significance were not included in this list of definite positive reactors. The type of ocular inflammation produced in the sensitized eye by the gastric introduction of the antigen differed from that produced by a similar intravenous injection only in the fact that the onset of the vascular reaction was usually retarded in the former group. In most instances the eye response was barely perceptible in the first 5 hours of the experiment and the maximum reaction appeared 12 to 18 hours later.

The chief antigen used was fresh egg white. Its use depended upon the fact that it had been the central sensitizing antigen in our previous work. There was an opportunity, however, to test out a small series of other proteins. Table I shows the reactions obtained in a varied series of animals. It is evident from these figures that rabbit eyes sensitized to foreign erythrocytes may exhibit vascular response following the intragastric introduction of the homologous antigen. The failure to induce similar reactions with guinea pig plasma and horse and sheep serum may be due to the few animals employed or to the fact that the latter had proven poorer sensitizing agents than red blood cells.

No attempt was made to prove the reaction specific since tests with

the intravenous shocking route had repeatedly shown that a striking specificity was maintained. However, two rabbits with eyes previously injured with glycerine and eight with anterior chambers sensitized to an *S. scarlatinae* filtrate failed to show any ocular response to the intragastric introduction of large amounts of fresh egg white.

No evidence of a striking desensitization to the specific protein could be demonstrated in the animals exhibiting ocular inflammation after this single gastric introduction of the antigen. Nearly all the rabbits showing a positive eye reaction experienced a similar response to the intravenous injection of the same antigen 48 hours later. One animal so treated died of typical anaphylactic shock.

TABLE I

The Effect Produced in Sensitized Rabbit Eyes Following the Introduction of the Specific Antigen into the Gastrointestinal Tract
Eye Reaction

Type of antigen	No. positive	No. negative
Fresh egg white	8	14
Guinea pig erythrocytes	1	8
Human erythrocytes	1	1
Cat erythrocytes	1	0
Guinea pig plasma	0	1
Horse serum	0	2
Sheep serum	0	1

A consideration of the data leads to the conclusion that the actively sensitized anterior chamber of the eye of the rabbit may become the site of a sterile inflammatory process following the introduction of the homologous antigen into the gastrointestinal tract. Clinical experience would certainly have suggested this possibility. The implications of this phenomenon are obvious. There are innumerable instances of asthma, hay-fever, and other types of hypersensitive response in which the precipitating factor has been proven to be an ingested protein. To Walzer's observation, therefore, that a *passively* sensitized local skin area may develop the typical hypersensitive response following the ingestion of the specific protein there must now be added the fact that a similar vascular response may be produced in an *actively*

sensitized rabbit anterior chamber following the intragastric introduction of the homologous antigen.

CONCLUSION

In the *actively* sensitized rabbit eye a sterile inflammation may develop after the introduction of the homologous antigen into the gastrointestinal tract.

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CRITERIA OF THE AGE OF LYMPHOCYTES IN THE PERIPHERAL BLOOD

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PLATES 25 TO 27

(Received for publication, May 5, 1931)

Unlike most of the other cells in the adult body, all those found normally within the circulating blood continue throughout life to develop from primitive forms. In any analysis of the blood in disease, it is of the greatest importance to be able to determine the state of maturity of the cellular elements. The present work is concerned with criteria of the age of the lymphocyte.

There are two principal sites of origin for the blood cells: the bone marrow and the lymph nodes. Most of our present knowledge concerning the maturation cycle and the relative age or youth of cells in the circulation has been derived from studies of the erythrocytes and granulocytes arising in the bone marrow. By noting variations in certain cytoplasmic (1, 2) or nuclear (3-6) criteria, the relative age of these cells can be estimated and valuable information be obtained. The studies thus far recorded of lymphocytes and lymphatic tissue have not been of a character to contribute materially to the establishment of acceptable criteria of maturation for this strain of white blood cells. A search of the literature reveals some attempts to link the so called lymphocyte with the other blood cells as a hemoblast or precursor in the development of the various well recognized definitive types. Arneth (7), following his work on the neutrophilic granulocyte, published an index of nuclear variation in the lymphocytes to which he attached maturative significance. His studies have not received the verification and acceptance accorded to his observations on the polymorphonuclear leucocyte.

There are several reasons why the search for recognizable criteria of maturation in the lymphocyte has not been pressed. One must assume to begin with that among the mononuclear cells called "lymphocytes," some, if not all, arise as immature lymphocytes (lymphoblasts), and mature in a definite orderly way, to degenerate eventually without dedifferentiation. This assumption involves indorsement, at least in part, of the phylogenetic doctrine of cell evolution in relation to

the lymphocyte, as it is obviously necessary for a cell to have a definite life cycle without change to other cell types before any conception of an orderly maturation can be entertained. The justification for such an assumption in relation to the lymphocyte will be evident as the experimental data which are to follow are introduced.

Another difficulty of the problem lies in the physical character of the lymphocyte itself, in that there is no apparent elaboration of specific structures in the cytoplasm. The development of granules in the cytoplasm and of nuclear polymorphism in the granulocyte, of a rosette of vacuoles stainable with neutral red in the monocyte, of phagocytic vacuoles without pattern in the clasmatocyte, and of hemoglobin in the red blood cell have all served as objective criteria upon which to base identification and a study of maturative phases. The lack of comparable criteria has been an obstacle to the recognition of age states in the lymphocyte, accustomed as we are to look for specific products in specific cells. It is evident that criteria of a different character must be sought for.

A third difficulty is to be found probably in the physiology of the lymphocytogenic tissues. Because of their anatomical distribution, it is highly probable that many of the lymphocytes perform their function within the tissue in which they arise. We may expect functionally mature lymphocytes to be intermingled with cells of varying degrees of immaturity in lymph nodes. It is impossible on the basis of present knowledge to examine lymphatic tissue, as in the case of bone marrow, with the assurance that there will be a preponderance of immature types with which to compare those forms in the circulating blood.

Finally, the uncertainty which exists concerning the exact structure of lymphatic tissue contributes to the complexity of the problem. Although most authors believe that the germinal center of Flemming is the lymphocytogenic focus, others interpret such so called germinal centers as reaction areas which do not participate in the origin of the lymphocyte.

Possible Criteria of Age

The following characteristics of blood cells may conceivably be of use in estimating the age of lymphocytes.

1. *Basophilia*.—In 1898 Pappenheim (8) noted that the cytoplasm of young myelocytes is very basophilic, and since then the association of basophilia with youth of the granulocytes has been noted by many investigators. The same fact has been observed for red blood cells. The "reticulation" in young red blood cells is merely a precipitation of basophilic substance (1, 2, 9-11); and in the reticulated cell counts we have an example of the use of the presence of basophilia as an index of immaturity. A careful application of the criterion to lymphocytes has apparently not been made.

2. *Mitochondria*.—That mitochondria are present in large numbers during the maturation of young blood cells other than lymphocytes is generally accepted (12–14); and in an excellent review, Cowdry (15) states that senescence is associated with diminishing numbers of mitochondria in the cytoplasm of cells in general. It should be mentioned, however, that increased numbers of mitochondria are associated with the activity of secretory cells.

3. *Size of the Cell*.—Hematologists have most frequently used cell size as a criterion of the age of lymphocytes. But how uncertain this has proved is shown by the fact that some have regarded the large (16, 17) and some the small (7, 18–20) lymphocyte as the younger cell, while others hold that size is no criterion of age at all (21). It is significant that most of the investigators studying fixed, stained specimens adhere to the belief that the large cells are the older types, whereas those who follow the supravital technique hold the reverse.

4. *Miscellaneous Features Characterizing the Lymphocyte*.—Here may be listed motility, vacuoles, chromatin content of nucleus, proportion of nucleus to cytoplasm, shape of nucleus (indentations), and azur granulation. The possible significance of these characteristics will be brought out further on.

Evaluation of Criteria

On analogy with the other cells of the blood, an estimation of the mitochondria in the supravital technique and of the degree of basophilia in the fixed films would seem most likely to afford criteria of value in the estimation of the age of the lymphocyte.

To test out this possibility, it is necessary to analyze the lymphocytes with respect to these criteria (1) in bloods which may be expected to show a normal distribution of age types, and (2) in bloods obtained when a physiological or pathological hyperplasia of lymphoid tissue should lead to increased numbers of immature types in the circulation. Of the criteria, basophilia is the most easily and accurately determined.

1. *Basophilia*.—With the supravital technique (17, 22) basophilia is detected by a grayish yellow hue or cast to the cytoplasm. In very basophilic cells this color approaches the hemoglobin tint of mature red corpuscles. A difficulty exists, however, in estimating relative basophilia in all the lymphocytes in supravital preparations, because of the very small amount of cytoplasm in the small

cells. Only rarely can this criterion be applied except in intermediate and large lymphocytes, and since the small cell usually predominates, the factor of basophilia cannot be analyzed uniformly in the supravital preparations.

Fixed and stained films furnish the best means for studying basophilia. Nearly all of the Romanowsky dye combinations bring out the basophilia clearly; but a combination of Wright and Giemsa stains (23) most clearly indicates its finer degrees. The usual Wright's staining method is followed, without washing, by a floating of the cover slip, stained side down, in diluted Giemsa (15 drops Giemsa plus 10 cc. distilled water at pH 6.5-7.0) for about 4 minutes. The cover slips are then blotted and mounted. The cytoplasm of lymphocytes usually does not take the stain uniformly; there may be condensations and rarefactions or an appearance of granulation. This is probably due to a precipitation of the basophilic substance comparable to the "reticulation" of red cells. Care must be observed to select fields for counting in which the spread is very thin, with no cells overlapping. It is also of prime importance that the stain be working properly, as it is useless to attempt to grade cells on basophilia in a poorly stained specimen.

After accustoming the eye to the differing shades of blue which this stain imparts to the cytoplasm, one can divide the lymphocytes arbitrarily into three types: The very basophilic, the moderately basophilic, and the slightly or not at all basophilic. The first type (Figs. 1 and 2) reacts with a color such as that obtained when a mark is made on white paper with a blue crayon (hereafter designated as "Y" types); the second type (Figs. 3 and 4) is "sky blue" (designated "M" types); while the third (Figs. 5, 6, and 7) is tinged scarcely at all with blue or may even react with a faint pinkish tinge to the cytoplasm ("O" types). Because of the gradual transition between the shades of color, it may be difficult at times to classify a cell, but with a little experience one learns to divide them rather sharply into the three classes. The smallest fragment of cytoplasm in a small lymphocyte is sufficient to make the classification on the basis of basophilia.

As the first step toward evaluating basophilia as a criterion of age, an analysis of the lymphocytes in a series of normal adult human and rabbit bloods was made. A total of 100 to 200 cells of all types were counted in the fixed smears and a percentage computation was made for each of the three degrees of basophilia. Tables I and II give the data procured.

In Table I the results obtained in blood specimen R 612C are omitted from the averages, as it was found that this man, an X-ray worker, was subject to recurring periods of lymphopenia, a fact discovered as a direct result of the finding in this survey.

It will be seen that normal bloods, both human and rabbit, have relatively definite and stable percentage composition of basophilic

lymphocyte types. The average formula (Y-M-O) which expresses decreasing degrees of basophilia is for 13 human blood samples, 5-49-46. For 14 normal rabbits this formula is 4.7-48-47.3. As the tables show, the individual variation from these formulae was remarkably little. The number of specimens studied is insufficient to enable one to accept the formulae as representative of normal physiological

TABLE I
Basophilia and Comparative Size of Lymphocytes in Normal Human Blood

Specimen	Sex	Date	No. of lymphocytes per c.mm.	Percentage of basophilic types Fixed technique			Percentage of size types Supravital technique		
				Y forms	M forms	O forms	Small	Int.	Large
R 1497*	F	May 21	1,768	9	48	43	96	4	0
R 1498	M	May 21	1,749	6	64	30	56	21	3
R 1499	M	May 21	869	5	55	40	88	12	0
R 1500	F	May 21	1,539	4	54	42	80	12	8
R 1501	M	May 21	2,240	6	47	47	82	15	3
R 1502	M	May 21	3,081	3	56	41	59	41	0
R 1503	M	May 21	4,238	4	65	31	98	2	0
R 169F	M	Apr. 25	3,360	7	34	59	72	28	0
R 612C	M	Apr. 25	896	17	58	15	100	0	0
R 612W	M	Apr. 25	1,716	3	36	61	76	24	0
R 612P	M	Apr. 25	1,470	6	69	25	70	30	0
R 612L	M	May 20	1,608	4	32	64	84	16	0
R 1493T	M	May 20	854	8	35	57	86	14	0
R 1493M	F	May 20	1,001	3	50	47	92	8	0
Average (R 612C omitted)			1,960	5	49	46	82	17	1

* These are serial numbers of the work of the department covering a term of years.

standards, but they are adequate for the purposes of the present investigation.

The second step was to examine bloods which might be expected to show an increase in immature lymphocytes. Since in new-born animals immature myeloid and erythroid cells are regularly found in the circulating blood, it is logical to expect an unusually great proportion of immature lymphocytes. Examination was made of 12 samples of blood from 6 rabbits the ages of which varied from 12 hours to 19

days, and the lymphocytes were graded according to basophilia as before. Owing to the extreme leucopenia in the young rabbits, only 25 to 50 cells of all types were counted. Table III contains the data thus secured.

Considering for the moment only the column containing the basophilic types, it is noted that the average formula (Y-M-O) is 38.8-

TABLE II

Basophilia and Comparative Size of Lymphocytes in Blood of Normal Rabbits

Rabbit	Date	No. of lymphocytes per c.mm.	Percentage of basophilic types Fixed technique			Percentage of size types Supravital technique		
			Y	M	O	Small	Int.	Large
R 1129*	3/12-4/14	3,045	6.7	47	46.3	92.6	6	1.4
R 1130**	3/12-4/21	6,079	3.7	50.6	45.7	90.7	7.8	1.5
R 1134**	3/12-4/14	2,623	11.1	54.4	34.5	87.8	8.6	3.6
R 1131	5/20	2,968	3	53	44	72	28	0
R 1132	5/20	13,778	2	56	42	99	1	0
R 1133	5/20	2,275	7	45	48	94	3	3
R 1135	5/20	4,033	15	56	29	88	9	3
R 1136	5/20	3,549	2	49	49	89	11	0
R 1137	5/20	2,460	2	50	48	83	14	3
R 1138	5/20	1,396	7	53	40	90	10	0
R 1139	5/20	7,864	3	44	53	98	2	0
R 1167	5/20	7,260	0	40	60	92	3	5
R 1169	5/20	4,902	0	26	74	94	6	0
R 1170	5/20	2,004	3	45	52	96	4	0
Averages.....		4,590	4.7	48	47.3	90.5	8.1	1.4

* R 1129 represents average of 9 counts.

** R 1130 and R 1134 represent averages of 11 counts each.

53.3-7.9; that is, there is a marked shifting to the heavily basophilic types as compared with specimens of normal adult blood.

Blood specimens from 20 rabbits with tuberculosis were next examined for possible changes in the cellular formula (Table IV). These tuberculous rabbits have been divided into two groups on the basis of the monocyte-lymphocyte ratio in the peripheral blood; eleven in which the M/L index was less than 0.50, probably indicating a minimum of tuberculous activity (24, 25), and nine in which this index

was 0.50 or more, probably indicating progressive lesions. In the latter group it will be seen that there is a notable shift in the lymphocyte formula to the more basophilic types, 22 per cent, together with a relative lymphopenia, when compared with the group in which the M/L index was less than 0.50. In those cases in which the M/L ratio is highest (see R 1366, R 1309, and R 1103, in particular), the increase in number of basophilic lymphocytes is especially marked.

TABLE III

Percentage of Basophilic, Mitochondrial, and Size Types in the Blood of New-Born Rabbits

Rabbit	Age	Date	No. of lymphocytes per c.mm.	Percentage of basophilic types Fixed technique			Percentage of mitochondrial types Supravital technique			Percentage of size types Supravital technique		
				Y	M	O	Y	M	O	Small	Int.	Large
R 1377	4 days	3/8	864	15	78	7	38	38	24	70	30	0
R 1377	10 "	3/15	1,100	50	46	4	0*	100	0	88	12	0
R 1377	19 "	3/24	2,400	43	53	4	42	53	5	84	14	4
R 1378	4 "	3/8	810	47	47	6	0*	70	30	90	10	0
R 1378	10 "	3/15	840	59	36	5	24	62	14	95	5	0
R 1378	19 "	3/24	605	32	63	5	29	61	10	90	5	5
R 1379	4 "	3/8	594	30	65	5	0	57	43	34	66	0
R 1379	10 "	3/15	592	35	53	12	25	69	6	94	6	0
R 1379	19 "	3/24	1,620	46	32	22	15	55	30	96	4	0
R 1418	12 hrs.	3/31	192	25	50	25	25	50	25	100	0	0
R 1419	12 "	3/31	320	44	56	0	42	58	0	88	12	0
R 1420	12 "	3/31	260	40	60	0	0*	100	0	100	0	0
Averages.....			850	38.8	53.3	7.9	20.0	64.5	15.5	85.7	13.6	0.7

* Note that the difficulty in accurately estimating large numbers of mitochondria sometimes results in many or all the "Y" types being classified with the "M" types. This remark also applies to Table VI.

In general, this relationship between basophilia and the M/L index persists throughout the table.

Since it is generally believed that the lymphocyte is an important element in resistance to tuberculosis, it is logical to assume that in cases progressing unfavorably, cases that is to say with a high M/L index, the lymphopenia reflects a demand for lymphocytes which the lymphatic tissues cannot adequately meet, and under such circumstances

the appearance in the blood stream of less mature types may be expected and is easily understood. In formulating this hypothesis, I am

TABLE IV

Percentage of Basophilic and Size Types of Lymphocytes in the Blood of Tuberculous Rabbits

Rabbit	M/L index	Date	No. of lymphocytes per c.mm.	Percentage of basophilic types Fixed technique			Percentage of size types Supravital technique		
				Y	M	O	Small	Int.	Large
R 1366	1.25	4/29	1,680	31	62	7	88	12	0
R 1309	1.21	4/21	2,590	53	41	6	93	7	0
R 1103	1.00	4/21	2,268	24	48	28	90	5	5
R 1152	0.93	5/19	2,380	13	60	27	84	16	0
R 1248	0.80	5/28	3,680	15	60	25	85	12	3
R 1262	0.60	4/28	4,865	9	52	39	94	3	3
R 1260	0.56	5/28	3,888	10	52	38	80	17	3
R 1381	0.56	4/28	1,927	18	53	29	90	5	5
R 1367	0.50	4/29	3,710	25	65	10	97	3	0
R 1383	0.43	4/28	3,220	11	57	32	83	17	0
R 1259	0.35	5/28	2,793	14	50	36	70	27	3
R 1112	0.33	4/30	3,663	13	38	49	76	24	0
R 1103	0.31	5/28	1,856	10	50	40	87	13	0
R 1184	0.22	5/28	2,808	13	44	43	91	7	2
R 1255	0.18	5/28	8,673	1	36	63	100	0	0
R 1389	0.18	4/28	3,753	11	63	26	93	7	0
R 1179	0.16	5/28	4,434	15	50	35	90	10	0
R 1269	0.15	5/28	3,054	5	52	43	93	7	0
R 1382	0.12*	4/1	5,964	26	60	14	60	30	10
R 1176	0.10	5/28	7,735	4	47	49	88	12	0
Average of all rabbits			3,747	16.0	52.0	31.9	86.6	11.7	1.7
Average of group with M/L index of 1.25-0.50 (9 animals).....			2,998	22.0	54.7	23.2	89.0	8.8	2.1
Average of group with M/L index of 0.43-0.10 (11 animals)...			4,359	11.1	49.7	39.0	84.6	14.0	1.3

* Rabbit R 1382 died 2 weeks after this blood count was taken, indicating that this animal probably had advanced tuberculosis.

merely applying to the lymphatic tissues the same reasoning which has been applied to the myeloid tissues and found true in the case of pyogenic infections.

Further evidence to show that increasing basophilia is a criterion of increasing immaturity was obtained by the examination of blood from animals in which lymphoid hyperplasia has been artificially induced. Figs. 10-15 show the degree of hyperplasia induced in the lymphatic tissues of rabbits receiving parenteral injections of foreign protein (26). They show a multiplication of lymph follicles both in lymph nodes and in the spleen, together with an increase in basophilic forms and in mitotic figures in these tissues. The blood counts of these same animals are given in Table V.

The left half of Table V shows the extent of increase in the numbers of lymphocytes per cubic millimeter during the period of the injections. The basophilic formula at various times during the period of the injections is shown in the right hand section of the table. Expressed as an average of 25 examinations of the blood from 5 animals, this formula is 27.1-46.9-26, which again shows a marked shift to the more basophilic types. If the hypothesis is correct that the peripheral blood reflects the state of activity of the tissue from which its cells are derived, then it must be concluded that basophilia is a measure of immaturity of the lymphocyte.

2. *Mitochondria*.—If it be accepted on the basis of the above evidence that basophilia is directly related to youth in the lymphocyte, then it becomes possible to see if any other property, such as a high mitochondrial content, is linked with youth.

Blood specimens in normal adult, new-born, and tuberculous rabbits were examined for the purpose of obtaining direct comparison between these two factors. The intensity of basophilia was determined in Wright-Giemsa-stained specimens and mitochondrial content in supravital preparations. To preclude obtaining specimens of blood for comparison that might be dissimilar with respect to the lymphocytes, both preparations were made from portions of the same drop of blood by drawing it into the stem of a white cell diluting pipette and expelling small portions very rapidly, first for the supravital and then for the fixed preparations.

Mitochondrial content was determined by count whenever possible,

at other times by rough estimation; and the classification was made as follows: Class Y represents cells with many mitochondria, that is, more than 20; Class M includes cells with from about 5 to 20 mitochondria,

TABLE V

Percentages of Basophilic Types of Lymphocytes in Rabbits with a Hyperplasia of the Lymphatic Tissues Induced by Protein Injections

Rabbit	Average Nos. of lymphocytes per c.mm. of blood during control and injection periods with date of autopsy		Date of count	Per cent basophilic types during injection period Fixed technique		
				Y	M	O
R 1157	Control period	2,477	2/21/30	27	51	22
	Injection period	3,911	2/26/30	59	30	11
	Date of autopsy	3/27/30	3/14/30	25	50	25
			3/19/30	27	59	14
			2/24/30	23	42	35
			3/26/30	24	39	37
R 1158	Control period	3,507	2/10/30	32	45	23
	Injection period	5,151	2/11/30	30	52	18
	Date of autopsy	2/21/30	2/19/30	33	38	29
			2/21/30	38	43	19
R 1160	Control period	3,179	2/26/30	37	43	20
	Injection period	5,599	3/ 7/30	20	51	29
	Date of autopsy	3/20/30	3/10/30	10	41	49
			3/14/30	23	40	37
			3/19/30	32	43	25
R 1163	Control period	3,208	2/14/30	20	57	23
	Injection period	6,500	3/ 7/30	16	60	24
	Date of autopsy	4/ 4/30	3/10/30	20	47	33
			3/19/30	18	54	28
			3/24/30	15	52	33
			3/26/30	31	47	22
			4/ 2/30	18	54	28
			4/ 4/30	13	46	41
R 1185	Control period	2,504				
	Injection period	5,983	4/25/30	62	28	10
	Date of autopsy	6/20/30	4/28/30	25	59	16
Averages....	Control period	2,975		27.1	46.9	26
	Injection period	5,429				

and Class O cells with from 0 to 5. The corresponding groups of lymphocytes arranged according to basophilia of the cytoplasm as determined in the fixed films were classified as before; that is, Class Y, deeply basophilic, Class M, moderately basophilic, and Class O, slightly or not at all basophilic. Tables III and VI present the data so obtained.

It is impossible to obtain accurate estimations of mitochondrial content, partly because of the physical difficulties involved in counting these bodies and partly because of the fact that some of the mitochondria occasionally fail to take the dye, while some fading of the stain often occurs. Tables III and VI are therefore presented to show trends, not exact quantitative relationships. As such, the averages in these tables demonstrate clearly that when the trend of the lymphocytes is towards increased cytoplasmic basophilia there is also a corresponding shift towards increased mitochondrial content. This same fact is demonstrated in Table VIII, Columns 6 and 7, in which basophilia and mitochondrial content of each of 75 large lymphocytes are directly compared by supravital examination.

3. *Size of the Cell.*—With the demonstration that increasing mitochondrial and basophilic content of the cytoplasm of lymphocytes are found together and that they are criteria of increasing immaturity, it becomes important to test the relation that cell size bears to these indices. The facts to be determined are: (1) The relation, if any, between increasing immaturity and the increase or decrease in numbers of the large cells, and (2) the determination of the status of the large lymphocyte with respect to its position in the maturative cycle. As a prerequisite for this study it was necessary first, to consider the measurements obtained as such, and second, to determine if the procedure of pulling and fixing cover slips distorts the size and appearance of the cell.

Among many hundreds of lymphocytes measured, the smallest encountered was 5.2 micra in diameter, and the largest 20.0 micra. The usual allocation of size to type has been given as 6μ – 9μ for the "small" lymphocyte, 9μ – 12μ for the "intermediate," and 12μ + for the "large" lymphocyte (16, 20, 21, 27). It was found, however, that a great number of cells, when studied by the supravital method, measured slightly over 9μ , with very few between this size and 10.5μ .

For this reason the following standards of size were adopted in the present study: up to 10μ the small lymphocyte, 10μ - 12μ the intermediate, and 12μ + the large lymphocyte.

TABLE VI

Comparison by Percentage of Mitochondrial and Basophilic Types of Lymphocytes in Three Normal and Five Tuberculous Rabbits

Rabbit	Date	Pathology	Percentage of basophilic types Fixed technique			Percentage of mitochondrial types Supravital technique		
			Y	M	O	Y	M	O
R 1129	3/12	None	7	50	43	3	55	42
R 1129	3/14	"	4	46	50	3	51	46
R 1129	3/17	"	10	44	46	6	73	21
R 1129	3/19	"	5	47	48	5	47	48
R 1129	3/24	"	7	57	36	7	57	36
R 1129	3/28	"	6	45	49	8	52	40
R 1129	3/31	"	10	37	53	4	61	35
R 1129	4/2	"	5	52	43	5	52	43
R 1130	3/12	"	3	60	37	20	60	20
R 1130	3/14	"	8	61	31	0	50	50
R 1130	3/17	"	2	48	50	6	60	34
R 1130	3/19	"	2	55	43	3	58	39
R 1130	3/24	"	5	56	39	3	65	32
R 1130	4/2	"	5	49	46	8	55	37
R 1134	3/12	"	11	46	43	18	43	39
R 1134	3/14	"	12	62	26	0	74	26
R 1134	3/17	"	20	47	33	9	69	22
R 1134	3/19	"	17	60	23	17	61	22
R 1134	3/24	"	16	43	41	8	60	32
R 1134	4/2	"	12	50	38	6	63	31
R 1185	4/25	Tbc.	62	28	10	24	62	14
R 1176	4/2	"	30	60	10	0	80	20
R 1366	4/29	"	31	62	7	18	75	7
R 1367	4/29	"	25	65	10	30	61	9
R 1382	4/1	"	26	60	14	11	60	29
Averages for R 1129.....			6.7	47.2	46.1	5.1	56.0	38.9
Averages for R 1130.....			4.1	54.8	41.1	6.6	58.0	35.4
Averages for R 1134.....			14.6	51.3	34.1	9.6	61.6	28.8
Averages for tbc. rabbits.....			34.8	55.0	10.2	16.6	67.6	15.8

Within limits, there is little or no relation between the size of the lymphocyte as obtained in the fixed smear and the real size of that cell in the circulating blood. Evidence in support of this statement is offered in Table VII. This represents a comparison of the measured gross diameters of the lymphocytes encountered in five separate differential counts of five different normal human bloods. Portions

TABLE VII

Number of Lymphocytes of Various Sizes Found in Counting 100 White Blood Cells in Supravital and Fixed Preparations of Blood from Five Normal Human Adults

Blood specimen	Technique	Gross cell diameters in micra																	
		5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14	14-15	15-16	16-17	17-18	18-19	19-20	20+		
R 1497 Female—age 58	Supravital	—	6	13	3	4	1	—	—	—	—	—	—	—	—	—	—	—	—
	Fixed	—	—	—	1	8	1	4	3	2	6	2	—	—	—	—	—	—	—
R 1498 Male—age 28	Supravital	—	—	6	8	11	6	1	1	—	—	—	—	—	—	—	—	—	—
	Fixed	—	—	—	4	5	4	10	7	0	2	1	—	—	—	—	—	—	—
R 1499 Male—age 31	Supravital	1	2	2	3	6	0	2	—	—	—	—	—	—	—	—	—	—	—
	Fixed	—	—	—	—	1	2	4	6	1	2	1	—	—	—	—	—	—	—
R 1500 Female—age 27	Supravital	—	3	9	1	8	1	3	1	0	1	—	—	—	—	—	—	—	—
	Fixed	—	—	—	1	3	4	10	6	0	2	1	—	—	—	—	—	—	—
R 1501 Male—age 33	Supravital	2	4	8	6	6	0	4	0	0	1	—	—	—	—	—	—	—	—
	Fixed	—	—	—	1	5	2	6	6	2	6	2	0	0	0	0	2	—	—
Totals	Supravital	3	15	38	21	35	8	10	2	0	2	—	—	—	—	—	—	—	—
	Fixed	—	—	—	7	22	13	34	26	5	18	7	0	0	0	2	—	—	—
Per cent	Supravital	83					13			4									
	Fixed	20					35			45									

from the same drop of blood were used in making both supravital and fixed spreads.

It is obvious that there is a great preponderance of large forms in the fixed films that does not occur in supravital preparations. Thus, only 4 per cent of the lymphocytes in the living state measured over 12 micra, whereas in the fixed films the corresponding percentage was 45.

This means, that in viewing a large lymphocyte in the fixed preparation, one cannot tell the size of that cell when in the circulating blood. The explanation is suggested that some cells, being more fragile than others, are flattened out by the technique usual in pulling cover slips with consequent distortion of size. Others, being more elastic and coherent, resist more effectually this mechanical factor. In cases of decrease in cell size, it can be supposed that contact with fixative has resulted in shrinkage of the cells.

Having determined that the size of cells in fixed specimens is not comparable with that of living ones, it became necessary to compare the percentages of the three cell sizes, as determined by supravital examination, with the percentages of the basophilic types obtained in fixed films, if one was to establish any relation between size and state of maturity. Such comparisons in bloods from normal human adults (Table I), normal adult rabbits (Table II), new-born rabbits (Table III), and tuberculous rabbits (Table IV) show that an increase in immature forms may occur without any notable shift in size. However, an analysis of these tables indicates that in some instances an increase in young forms was associated also with an increase in the larger types of cells (compare average of size types in Tables II, III, and IV and individual counts of R 1377 and R 1378 on March 24 of Table III, and R 1382 on Table IV).

A study of large lymphocytes was now undertaken to determine if they are predominately young or old. To determine this point, 75 large lymphocytes were measured in supravital specimens and observations were also made on their degree of basophilia and mitochondrial content and on certain other features of the living cell. The results obtained are shown in Table VIII, Columns 2, 6, and 7.

Analysis of Table VIII indicates that most but not all of the large lymphocytes have a marked basophilia and contain large numbers of mitochondria. Those that are deeply basophilic and with nearly maximum numbers of mitochondria (+++ and ++++) make up 70 per cent of the large lymphocytes examined, whereas the cells deficient in these respects (0 and 1+) constitute only 15 per cent of those surveyed. As an example of the latter group, a very large cell (No. 27), measuring 16.1μ , had a cytoplasm entirely deficient in signs of basophilia and mitochondria. Since these two characteristics,

TABLE VIII

Supravital Characteristics of Large Lymphocytes in the Blood of Rabbits

Cell No.	Gross diameter	Approximate ratio of diameter of nucleus to cell	Chromatin content of nuclei	Approximate depth of indentation of nucleus	Basophilia of cytoplasm	Mitochondria	Neutral red vacuoles	Nucleoli	Non-staining refractive vacuoles
				μ					
1	14.9	0.90	++	F	++++	++++	+	2	0
2	13.3	0.60	++	F	++++	++++	++	0	0
3	15.0	0.70	++++	1.6	+++	+++	+	0	0
4	15.0	0.55	++++	Round	++++	++++	++	0	0
5	14.5	0.60	+++	2.6	++++	+++	+	0	0
6	16.6	0.75	++	1.8	++++	++++	++	0	0
7	15.0	0.70	++	1.2	+++	++	+	0	0
8	17.5	0.60	++++	0.5	++++	++++	++	0	0
9	14.5	0.70	++	0.5	++++	+++	++	0	0
10	15.0	0.80	++++	1.0	+	++	+++	0	0
11	13.2	0.55	++	1.6	+++	++	++	0	0
12	15.7	0.60	++	0	++++	+++	+	0	0
13	12.8	0.60	+	1.6	++++	++	+++	0	0
14	15.0	0.88	++++	Round	+++	+++	++	0	0
15	16.0	0.70	+	Round	++++	+++	+++	0	0
16	15.0	0.70	++	F	++++	++++	+	1	0
17	14.5	0.65	++++	2.2	++++	++++	++	0	0
18	13.0	0.75	+	1.5	+++	+++	++	0	0
19	13.0	0.75	++++	0	+++	+++	++	0	0
20	13.0	0.75	++++	F	+++	+++	++	0	0
21	16.0	0.60	++++	Oval	++++	++++	++	0	0
22	13.0	0.60	+	F	++	++	+	0	0
23	15.0	0.75	++	5.0	+++	++	+	0	0
24	14.0	0.70	++	4.0	++++	+++	++	0	0
25	19.5	0.90	++	4.5	++++	++++	+++	0	0
26	14.4	0.75	+	Round	++	++	+++	0	0
27	16.1	0.30	+++	Round	0	0	0	0	1
28	15.0	0.70	++	F	++	++	+	0	0
29	14.5	0.75	++	5.0	+++	+++	++	0	0
30	16.0	0.70	++	0.5	++++	++++	+++	0	0
31	14.5	0.70	++	2.0	+++	+++	+	0	0
32	14.4	0.45	+	Round	0	0	++	0	++++
33	15.0	0.50	++	F	+	+	++++	0	+
34	20.0	0.75	++	Oval	++++	++++	+++	0	0
35	16.5	0.95	++	Round	++++	++++	+++	1	0
36	13.0	0.85	++	Round	++++	+++	0	0	0
37	16.0	0.90	++	F	+++	+++	+++	0	0
38	14.5	0.50	+	Round	0	0	0	0	++++

F = flattened.

TABLE VIII—*Concluded*

Cell No.	Gross diameter	Approximate ratio of diameter of nucleus to cell	Chromatin content of nuclei	Approximate depth of indentation of nucleus	Basophilia of cytoplasm	Mitochondria	Neutral red vacuoles	Nucleoli	Non-staining refractive vacuoles
				μ					
39	16.0	0.75	+	Oval	++	++	++++	0	0
40	16.1	0.55	+	2.5	++	++	++++	0	0
41	19.0	0.80	++++	F	++++	++++	+++	0	0
42	14.4	0.90	++	1.0	++++	++++	++	0	0
43	15.0	0.60	++	Oval	+++	+++	++	0	0
44	14.5	0.70	++++	1.0	+++	+++	++	0	0
45	13.5	0.85	+++	Oval	++++	++++	+	0	0
46	15.0	0.75	+	F	+++	++	++	0	0
47	13.0	—	—	—	++++	++++	0	0	0
48	13.5	0.70	++	5.0	++	++	++++	0	0
49	16.0	0.55	+++	4.0	++++	++++	0	0	0
50	16.0	0.60	+	4.0	++	++	+	0	0
51	13.0	0.60	++	Oval	++++	++++	+	0	0
52	13.0	0.70	++	F	++	++	++	0	0
53	13.0	0.60	++	F	+	+	++++	0	++++
54	13.0	0.70	++	5.0	++	++	++++	0	0
55	15.0	0.65	++	2.0	++	++	++++	0	0
56	13.0	0.70	++	Round	++++	++++	+++	0	0
57	12.8	0.70	+	F	0	0	++++	0	0
58	15.0	0.70	++	F	++++	++++	+++	0	0
59	12.8	0.80	++++	Round	++++	++++	0	1	0
60	14.5	0.55	+++	Round	0	0	++	0	+
61	12.8	0.70	++	Oval	++++	++++	0	0	0
62	17.5	0.65	+++	Round	+++	+++	++	0	0
63	12.0	0.70	++	Oval	++	++	++++	0	0
64	16.0	0.75	++	Oval	++++	++++	+	0	0
65	14.5	0.70	++	F	+++	+++	++++	0	0
66	14.5	0.75	++	F	+++	+++	++++	0	0
67	14.5	0.60	++	3.0	+++	+++	++++	0	0
68	14.5	—	—	—	++++	++++	+	0	0
69	13.0	0.70	+++	Oval	+	+	++++	0	++
70	11.2	0.60	+++	Round	0	0	+	0	++++
71	14.5	0.60	+++	F	++	++	++	0	0
72	13.0	0.65	+	Oval	+	0	0	0	++
73	14.4	0.70	++	Oval	+++	+++	++	0	0
74	14.5	0.90	++++	Round	++++	++++	++	1	0
75	16.0	0.70	++++	Round	+++	+++	++	0	0

Cell 69 changed to Cell 70 in 15 minutes under observation.

Cell 72 was watched for 1½ hours and no change in morphology occurred.

when present, have been shown to be associated with immaturity, it can be concluded that, although the majority of large lymphocytes of the supravital films are young, cells may be large at any age. The same holds true for the erythrocyte and leucocyte.

4. *Miscellaneous Features Found in Lymphocytes.*—Amongst the features detectable in the lymphocytes of supravital preparations, there remain to be evaluated the shape of nucleus (indentations), the relative size of nucleus to gross cell size, chromatin content of the nucleus, non-staining refractive vacuoles, and content of neutral red vacuoles. For the association of these features with mitochondrial content, basophilia, and cell size, reference is again made to Table VIII. The number of neutral red bodies, mitochondria, and refractive vacuoles were counted or estimated on a basis of 0 to 5 = +; 5 to 15 = ++; 15 to 30 = +++; over 30 = +++++. The amount of indentation of nucleus was measured or estimated; basophilia and amount of chromatin in the nucleus were estimated as +, ++, +++, and +++++.

Analysis of Table VIII with respect to these features indicates:

- (a) That the shape of the nucleus of the lymphocyte (presence of indentation and its degree) does not bear any relationship to the number of mitochondria or the degree of basophilia, and hence is unrelated to age in direct contradiction to Arneth's hypothesis (7), whereas the chromatin content of the nucleus does appear to be greatest in those cells deficient in the cytoplasmic criteria of youth.
- (b) That in general, in the large lymphocytes, the greater the amount of cell occupied by the nucleus, the more basophilic the cytoplasm.
- (c) That nucleoli are visible only in the nuclei of cells with deeply basophilic cytoplasm and sparse chromatin. It is probable that only large nucleoli are visible by this method of examination, as very small nucleoli are likely to be obscured from view by nuclear substance.
- (d) That non-staining, refractive vacuoles are found only in cells devoid of both mitochondria and basophilic substance; whereas the presence and number of neutral red vacuoles do not seem to bear any relationship to either of these features.

Azur granulation is seen in fixed preparations, but not in the supravital. The observations made in the present study have indicated that these granules are associated most often with the lesser basophilic

types. However, one cannot be too certain that they are entirely limited to this older cell type, because the dark staining of the cytoplasm in the young basophilic cells may make it impossible to detect them when present. The fact that no cytoplasmic structure of the living lymphocyte can be identified with the azur granule of the dead cell suggests that the latter is a precipitation brought about by fixation, possibly evidencing some functional activity of the mature cell.

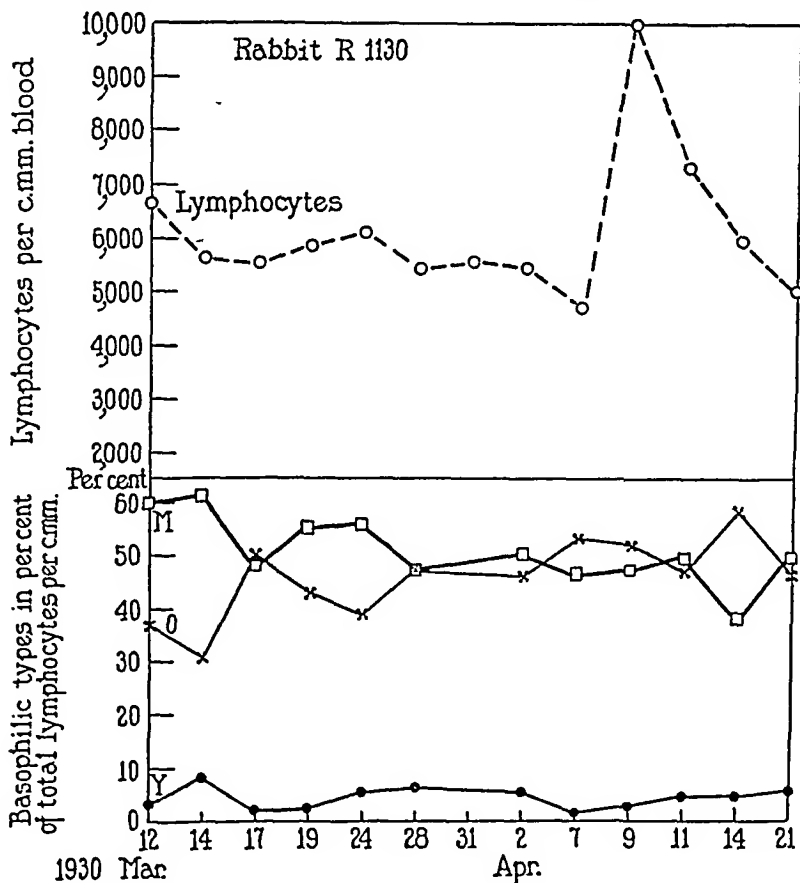


CHART 1. There are four curves on this chart to be contrasted. The uppermost shows the fluctuations in total number of lymphocytes; the curve labelled "O" designates the percentage of old cells (least basophilic); "M" refers to the mature (moderately basophilic) types; while the curve "Y" at the bottom of the chart indicates the percentage of young or heavily basophilic types.

Relation of Lymphocytosis to Cellular Immaturity

Armeth's dictum that the total number of granulocytes in the blood is not necessarily a criterion of the activity of the bone marrow has

been amply confirmed. It is of interest to analyze the number of lymphocytes circulating in the peripheral blood as an index to activity of the lymphoid tissue. Since it is now evident that the greater the basophilia the greater the immaturity of lymphocytes, one is enabled to make the analysis mentioned by comparing directly the percentage of the basophilic types with the total numbers of circulating cells. For this purpose, observations were made three times per week for 1 month on the lymphocytes in the blood of three stock rabbits. Chart 1 represents the findings in one of these animals. It is typical of all three.

Chart 1 shows clearly that the total number of lymphocytes in the peripheral blood is not necessarily a criterion of a percentage increase therein of young cells. Note especially that the high peak in total lymphocytes on April 9th showed no corresponding fluctuation in the percentage of basophilic types.

DISCUSSION

The present study shows that in the maturing lymphocyte changes take place in number of mitochondria and degree of basophilia, which have the same general character as those occurring during the life of the other cells of the blood.

Although basophilia is manifestly a criterion of youth, the absence of it in a given cell does not necessarily mean that the cell is mature. No one criterion enables one to pronounce upon this point. In other cell strains some of the recognized characteristics of youth may be absent in elements of undoubted immaturity. For example, occasionally there are seen early myelocytes that are motile (about 60 per cent in one case of myelocytic leucemia recently observed); mature neutrophilic leucocytes with very few specific granules; myelocytes without appreciable numbers of mitochondria or with little basophilic substance. From this experience with cell types other than the lymphocyte, it would seem that in the case of the lymphocyte as in that of other cell types, all of the criteria of immaturity must be taken into account in an age classification. Basophilia is the most constant and reliable criterion.

It now seems certain that each strain of blood cells can be traced back to a simpler form characterized by a more basophilic cytoplasm.

The widespread distribution throughout the body of basophilic mononuclear cells gave rise to Pappenheim's concept of a "lymphoidocyte" or indifferent polyvalent primitive cell. The monophyletic theory of cell origin has been based upon this conception. Although the decreasing basophilia which characterizes the cytoplasm of the lymphocyte from youth to senility is wholly analogous to that seen in the other types of developing white blood cells, nevertheless the demonstration of an early basophilic form in this series, need not necessarily be interpreted as support for the monophyletic hypothesis.

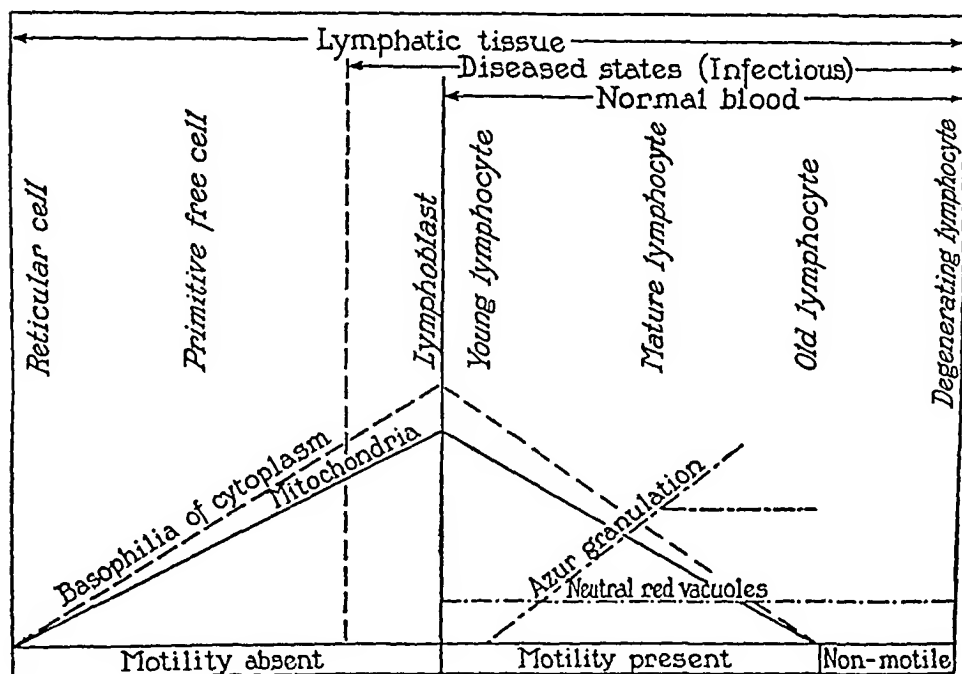


CHART 2. Diagram of the maturation of lymphocytes

The dualists have always held that each cell strain has its own "cytoblast," which matures only in the lineage peculiar to that specific cell type, while the monophyletic school have given the "lymphocyte" at all times multipotential powers of differentiation. The recognition of lessening basophilia of the lymphocyte until in the degenerating form the cytoplasm shows no basophilia whatever, establishes the lymphocyte as an independent definitive cell strain existing in parallel with the other blood cells, not preliminary to them. It will be recalled

that such an assumption was necessary to the undertaking of the present work; and it has been justified. A graphic representation of the changes taking place during the life of the lymphocyte is now possible. Chart 2 is such a representation, patterned after the schema of Sabin (13) for the red cells and granulocytes.

The failure to recognize basophilia as an expression of youth of the lymphocyte has often resulted in a designation of young forms as plasma cells. All possible gradations in basophilia have been encountered within the lymphocytic series as is indicated by Chart 2.

Unna's original concept (28) of the plasma cell included numerous basophilic forms. Von Marschalkó (29), however, sharply limited this term to a specific group of cells showing a relatively small nucleus excentrically placed, containing chromatin particles usually arranged radially (*Radkern*), with a central clear area in the protoplasm opposite the nucleus, and with very basophilic cytoplasm. The irritation forms of Türk (18) are usually considered as plasma cells (Naegeli (21)), but obviously if one accepts the definition of von Marschalkó these cells cannot be plasma cells.

Arneth's expression "shift to the left" (4) had its inception in the observation that under pathological conditions there occurs an increase in the proportion of those granulocytes showing fewer lobes to the nucleus, *i. e.*, younger cells. In a larger sense, "shift to the left" has come to express a shift toward the younger stages in the maturation cycle of some other blood elements, and applied in this broader sense, it is highly useful. When there occurs an increase in the proportion of older forms, the term "shift to the right" is applicable. It is evident from the present work that the terms can be used with respect to the lymphocyte.

SUMMARY

The study of blood from rabbits with normal and with hyperactive lymphatic tissue reveals, in the latter, a greater percentage of lymphocytes with heavily basophilic cytoplasm and numerous mitochondria. This indicates that cytoplasmic basophilia and mitochondrial content can serve as criteria of the degree of maturity of the lymphocyte, these characters having the same significance in this relation as obtains with other blood cells. Basophilia is the more evident and reliable indicator of youth of the cells. The classification of lymphocytes into

three groups, according to degree of basophilia, has yielded figures which show the proportions of the three to be relatively stable in blood from normal adult human beings and rabbits.

Size is not strictly a function of age in lymphocytes. Moreover, there is no correspondence in the size of lymphocytes in supravital films and in fixed specimens obtained by the "cover glass" method. There is a change of size during fixation. Although lymphocytes of intermediate and large size may be of any age, in supravital preparations the majority are young cells, whereas in fixed films the reverse obtains. The small lymphocyte may be of any age in specimens examined by either technique.

The total number of lymphocytes circulating at any given time is not necessarily an index to lymphoid activity.

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EXPLANATION OF PLATES

PLATE 25

FIG. 1. A large, very young lymphocyte from the peripheral blood of Rabbit R 1185, Apr. 25, 1930. Wright-Giemsa stain. Observe the granular character and extreme degree of basophilia of the cytoplasm. This is the "Y" type of cell mentioned in the text. Diameter of cell was 16.0 μ .

FIG. 2. A small lymphocyte showing the same characters. From Rabbit R 1381, Apr. 28, 1930. Diameter of cell was 8.5 μ .

FIG. 3. An intermediate lymphocyte from the peripheral blood of Rabbit R 1185, Apr. 25, 1930. Wright-Giemsa stain. Note the color of the cytoplasm. This is the "M" type of cell described in the text. Diameter of cell was 11.5 μ .

FIG. 4. A large lymphocyte showing the same features. From peripheral blood of Rabbit R 1185, Apr. 25, 1930. Diameter of cell 16.0 μ .

FIG. 5. A large lymphocyte from the peripheral blood of Rabbit R 1152, May 19, 1930. This is an old cell and is designated as the "O" type in the text. Note the lack of basophilia of the cytoplasm. One azur granule is to be seen near the top of the cell. Wright-Giemsa stain. Diameter of cell was 16.0 μ .

FIG. 6. An intermediate lymphocyte showing the same features. From peripheral blood of Rabbit R 1152, May 19, 1930. Diameter of cell was 11.8 μ .

FIG. 7. A small lymphocyte showing the same features. From the peripheral blood of Rabbit R 1176, May 28, 1930. Diameter of cell was 8.0 μ .

FIG. 8. A red blood cell from the peripheral blood of a rabbit stained by the Wright-Giemsa technique,—for purposes of comparison.

PLATE 26

FIG. 9. Section of inguinal lymph gland of Rabbit R 1160. Removed at biopsy before protein injections. Normal gland throughout. Hematoxylin and eosin. $\times 27$.

FIG. 10. Section of inguinal gland of Rabbit R 1160 after 45 daily intravenous injections of chick embryo extract, showing an increase in size and number of secondary nodules and an increase in internodular lymphatic tissue. Compare size and histology of this gland with that of Fig. 9 and note cellular response in the former. Hematoxylin and eosin. $\times 27$.

FIG. 11. Section of inguinal gland of Rabbit R 1158 after daily subcutaneous injections of chick embryo extract. The very hyperplastic gland consisted largely of diffusely distributed lymphatic tissue. This was rich in lymphoblasts with heavily basophilic cytoplasm. Hematoxylin and eosin. $\times 27$.

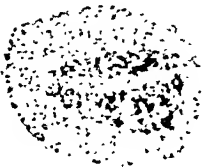
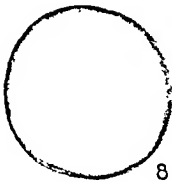
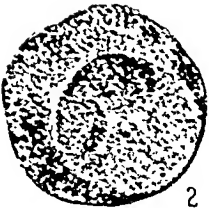
PLATE 27

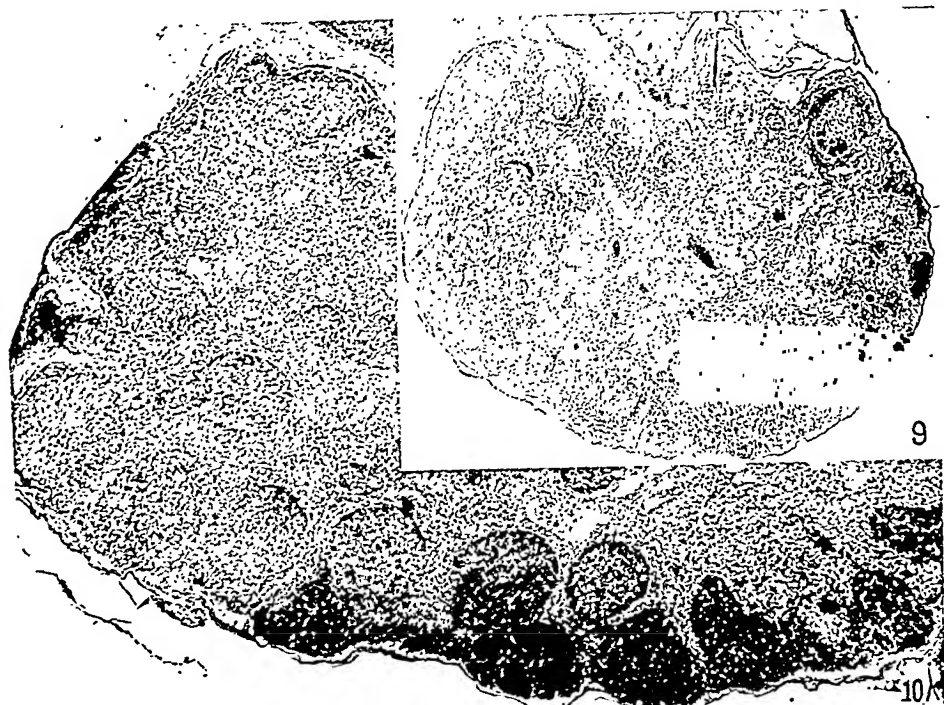
FIG. 12. Section of spleen of control Rabbit R 1186 after 37 daily intravenous injections of normal salt solution. No evidence of hyperplasia. Hematoxylin and eosin. $\times 27$.

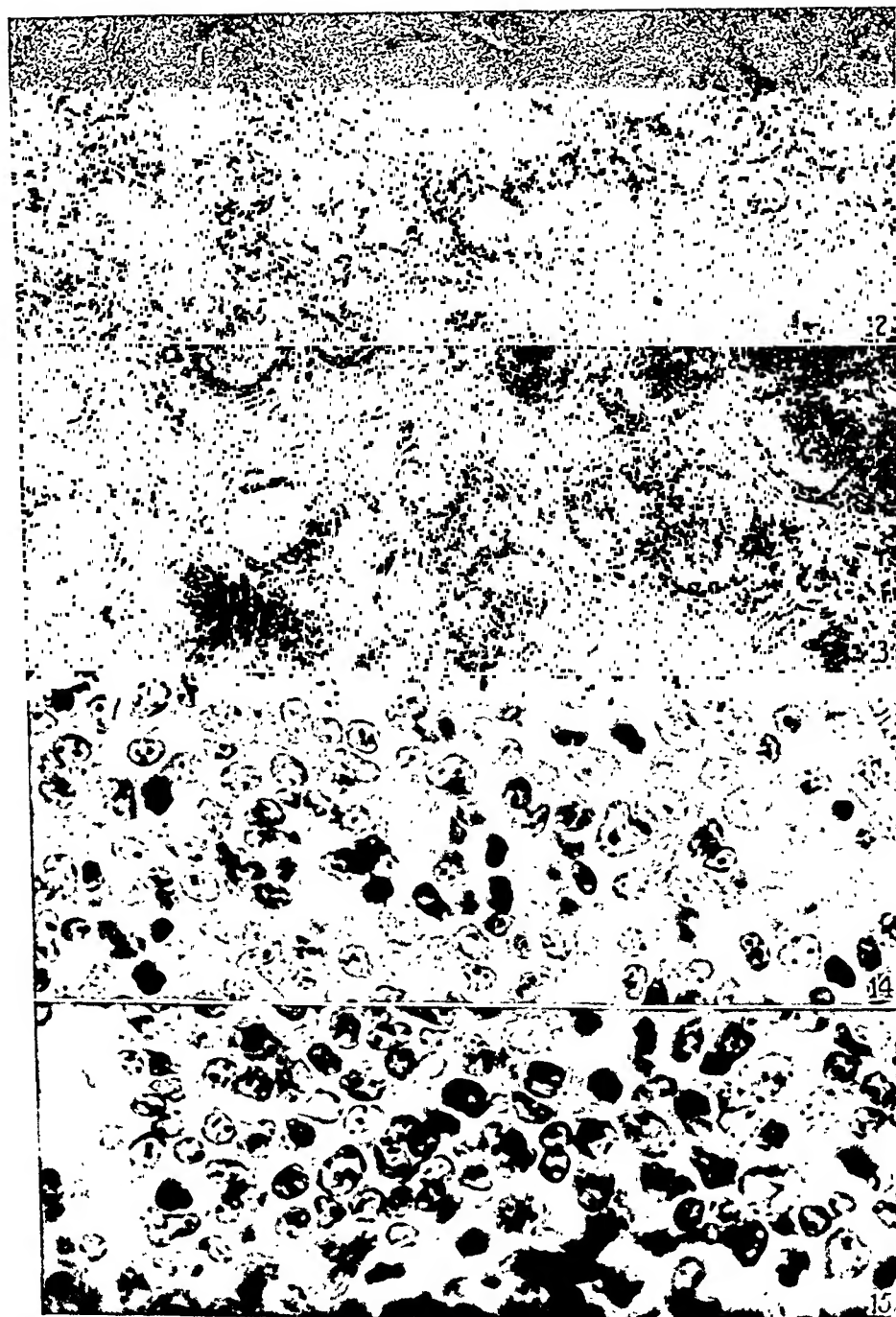
FIG. 13. Section of spleen of Rabbit R 1157 after 19 daily intravenous injections of chick embryo extract. Note the increase in size and number of Malpighian bodies as compared with control spleen of Fig. 12. Hematoxylin and eosin. $\times 27$.

FIG. 14. Section of spleen of Rabbit R 1163 through germinal center. This animal received 25 daily intravenous injections of a solution of egg albumen. Note increase in number of mitotic figures and large cells with basophilic cytoplasm, typical of the spleens from all rabbits receiving these injections. Hematoxylin and eosin. Oil immersion.

FIG. 15. Section of spleen of Rabbit R 1160 through germinal center. This animal received 45 daily intravenous injections of chick embryo extract. Note evidences of hyperplasia as in Fig. 14. Hematoxylin and eosin. Oil immersion.







Photographed by Louis Schmidt

(Wiseman: Age of lymphocytes in peripheral blood)

ON THE SPECIFICITY OF SEROLOGICAL REACTIONS WITH SIMPLE CHEMICAL COMPOUNDS (INHIBITION REACTIONS)

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Whereas the usual serological reactions involve the use of high-molecular antigens of unknown constitution, a method has been described in a previous paper (1) which permits of the application of serological reactions to compounds of simple chemical composition. The method is based upon the possibility of partially synthesizing antigens (2, 3), by attaching to proteins chemical substances of simple structure as can be done by coupling with diazo compounds. Immune sera produced by injecting such "synthetic antigens" exhibit a specificity depending on the simple substances forming a part of the complex, particularly if the test antigen used contains a protein different from that used for immunization.

In ordinary precipitin tests, it has long been noticed that the reactions can be inhibited by addition of an excess of the antigen. Applying this observation to artificial complex antigens, it has been shown that the precipitin reactions of these substances are often inhibited specifically by the addition of a sufficient quantity of the same or a similar chemical substance to that used in building up the antigenic complex (1). These observations have since been confirmed by Klopstock and Selter (4), Avery and Goebel (5), and by ourselves.

The inhibition reactions with substances of known chemical structure can be used for the study of serological specificity in general, and in this regard they have certain advantages over the precipitin or complement fixation tests made with the full artificial antigens. Thus, in the first place it is not necessary to use substances which can be combined with proteins and, consequently, a greater variety of compounds can readily be subjected to the test. Furthermore an advantage may arise from the circumstance that the possible

influence of the protein part of the antigen, and of the mode in which the specific part is attached, are entirely excluded.

A particular application of the inhibition test has been suggested previously (1), namely the determining of the nature of the specific group in an antigen in cases in which this is not known. This plan has been followed in our laboratory by Wormall (6), who was able to demonstrate that in iodized protein the reacting group is the diiodo-tyrosine radical, since the precipitin reaction of the protein can be inhibited specifically by diiodotyrosine but not by ortho-iodophenol or potassium iodide. An essentially similar method has repeatedly been employed in recent studies on the specificity of ferments.

In the present paper, some additional results are discussed which were obtained in the course of serological studies on azoproteins.

Influence on the Specificity of the Position of Substituents in Aromatic Compounds

The significance of the position of substituents in the benzene ring for the specificity of precipitin reactions was established in tests (2) with immune sera, reacting on antigens containing various aromatic acids, e.g., *o*-, *m*-, and *p*-aminobenzoic acid antigens.*

Similar conditions appeared to prevail in the inhibition tests. In fact, by means of an immune serum for *p*-aminobenzoic acid it was possible to differentiate various ortho- and para-monosubstituted benzoic acids, regardless of the nature of the substituent which was variously OH, CH₃, Br, Cl, or NO₂ (1). For, in all instances, solutions of the sodium salts of the *p*-acids were much more active than those of the *o*-acids, while the *m*-substituted compounds were intermediate in activity.

In order to obtain further information it seemed necessary to examine also immune sera for *o*- and *m*-substituted antigens which had not been studied sufficiently in the previous experiments. The antigens used for immunization were prepared by coupling diazotized *o*-, *m*-, and *p*-aminobenzoic acids with horse serum. For the technique of the prep-

* For the sake of brevity, the immune sera and antigens prepared with azoproteins will be designated by the compounds used for diazotization and subsequent coupling.

aration of the antigens, and the immunization, we refer to previous communications (1, 7).

For the inhibition tests 1 millimol of each substance to be tested was dissolved in water by the aid of sodium hydroxide, and the solution made neutral to litmus and brought up to a volume of 10 cc. Unless otherwise stated, in each test 0.05 cc. of this solution was mixed with 0.2 cc. of the diluted antigen (made with chicken serum) previous to the addition of the immune serum. The control tube (C) contained only antigen and immune serum. The dilutions of the antigens are in terms of a 5 per cent stock solution. The intensity of the precipitin reaction is indicated as follows: 0, f. tr. (faint trace), tr. (trace), tr. (strong trace), \pm , \pm , \pm , $\pm\pm$, etc.

The results in Table I are what would be expected from former findings, that is, comparing *o*- and *p*-immune sera and the *o*- and *p*-substances, the inhibition was always more intense with the "homologous" substance. Also the inhibition of the precipitation by the *m*-aminobenzoic immune sera was in general strongest with meta-substituted compounds, though in the case of the oxybenzoic acids the inhibition effect of the ortho-substance was somewhat greater than that of the meta-substituted acid. On comparing *o*- and *p*-derivatives of benzoic acid, it was seen moreover that substitution in the "heterologous" position often diminishes considerably the inhibition reaction as compared not only with the "homologous" substance, but also with the non-substituted benzoic acid.

An influence of greater or lesser strength of the acids seems to be excluded as the cause of these effects, because the regularities observed are of the same sort for substituted acids which differ widely in their strength, and also because of the results with the *m*-substituted substances. As suggested previously (1, 2), the most probable explanation is that the outcome of the reactions in the cases under consideration depends on the steric correspondence in the position of the substituents in the immunizing antigens and the compounds employed for inhibition, even though the substances examined differ in their composition from the specifically binding groupings in the antigens.

A relationship of the observations described to the phenomenon of ferment specificity is indicated by the findings of Waldschmidt-Leitz and Balls (8), who reported that of the isomer (chloroacetyl-amino) benzoic acids only the meta-compound is hydrolized by pancreatic

carboxypolypeptidase, a fact which the authors also explain on the basis of steric configuration. Other similar instances are the inhibition of the action of tyrosinase by aromatic acids (9), and the oxidation of various substrates by this enzyme (10). It is noteworthy that a specificity depending on the position of aromatic substituents has been observed recently in the skin reactions of a patient sensitive to resorcinol (meta-dihydroxybenzene) by Nathan and Stern (11). This patient did not react to either ortho- or para-dihydroxybenzene, nor to the methyl ethers of resorcinol, nor to phenol or pyrogallol.

Not all sera are equally well suited for the demonstration of the inhibitory effects. Thus with some sera which fell off after long storage and also with some fresh sera, the inhibition by benzoic acid was weaker than usual and the regularities described were blurred. In some such instances it was possible to increase the inhibition by heating the immune sera for 1 hour at 55°C. or by setting up the tests at that temperature.

Inhibition Effects by Fatty and Cyclic Acids

In the tests described previously (1), the reaction of a serum for *p*-aminobenzoic acid antigen was found to be inhibited by a variety of aromatic acids, *i.e.* benzoic acid and substituted benzoic acids, but not distinctly by several aliphatic acids tested. Since benzoic acid caused practically no interference with the precipitin reaction by sera for *p*-arsanilic acid or *m*-aminobenzenesulfonic acid—which, in turn, are inhibited by aromatic arsinic or sulfonic acids—it is plain that the effect produced by benzoic acid depends on the constitution of the substance containing a carboxyl group bound to the benzene ring, a structure which corresponds to that of the binding group in the antigen. In recent experiments it was, however, found that aliphatic carbonic acids, especially the higher ones such as caproic and heptylic acids, also inhibit the reaction of immune sera for aminobenzoic acids.

As Table II shows, the effect increased with the length of the chain and was most marked with the caproic and heptylic acids. Acids with much longer chains could not be properly tested on account of the turbidity of the neutral solutions, but from a test with caprylic acid it would seem that the action may be stronger with the higher members of the series. Consequently it is possible that the phenomena, at least in part, are due to the physicochemical properties of high fatty

TABLE II

The following substances were used for the inhibition tests: (1) benzoic acid; (2) cyclohexane carboxylic acid; (3) acetic acid; (4) propionic acid; (5) normal butyric acid; (6) isobutyric acid; (7) normal valeric acid; (8) isovaleric acid; (9) methylacetic acid; (10) normal caproic acid; (11) isocaproic acid; (12) diethylacetic acid; (13) normal heptylic acid.

Immune serum for	Serum No.	Reading after	1	2	3	4	5	6	7	8	9	10	11	12	13	C
			0	0	+	+	+	+	+	+	+	tr.	±	+	±	++
Ortho-aminobenzoic acid	5	15 min. 1 hr.	0	+	+	+	+	+	+	+	+	+	±	+	+	++
Para-aminobenzoic acid	6	5 min. 1 hr.	0	0	+	+	±	+	±	+	+	tr.	±	+	f. tr.	++
			0	+	+	+	+	+	+	+	+	+	+	+	+	++

TABLE III

The following substances were used for the inhibition tests: (1) benzoic acid; (2) α -thiophene carboxylic acid; (3) nicotinic acid; (4) cinchonic acid; (5) furan carboxylic acid; (6) picolinic acid; (7) cyclohexane carboxylic acid; (8) α -pyrrole carboxylic acid; (9) α -naphthoic acid; (10) β -naphthoic acid; (11) acetic acid; (12) normal caproic acid; (13) normal caprylic acid.

Immune serum for	Serum No.	Reading for	1	2	3	4	5	6	7	8	9	10	11	12	13	C
			0	0	tr.	0	tr.	±	f. tr.	tr.	0	0	+	tr.	f. tr.	+
Ortho-aminobenzoic acid	1	15 min. 1 hr. 2 hrs.	0	0	±	0	±	+	tr.	tr.	0	0	+	±	tr.	+
Meta-aminobenzoic acid	2	15 min. 1 hr. 2 hrs.	0	f. tr.	tr.	tr.	tr.	±	±	+	0	0	+	±	+	+
			f. tr.	tr.	tr.	tr.	tr.	+	+	+	0	0	+	+	+	+
Meta-aminobenzoic acid	3	15 min. 1 hr. 2 hrs.	0	f. tr.	tr.	±	±	+	±	+	0	f. tr.	+	+	±	+
			tr.	tr.	±	+	+	+	+	+	f. tr.	tr.	+	+	+	+
Para-aminobenzoic acid	4	15 min. 1 hr. 2 hrs.	f. tr.	0	tr.	f. tr.	tr.	±	tr.	±	f. tr.	tr.	+	+	±	+
			tr.	f. tr.	±	tr.	±	±	±	+	f. tr.	tr.	+	+	±	+
			tr.	tr.	±	±	±	±	±	+	tr.	tr.	+	+	±	+

acids. In this connection it should be mentioned that salts of cholic and desoxycholic acids appear to inhibit precipitin reactions in general. On the other hand, the chemical structure, particularly the presence of the carboxyl group, must play a part, since other precipitin reactions, *e.g.* that of an immune serum for arsanilic acid, are only slightly influenced by caproic or heptylic (or benzoic) acids. The fact that these acids have very little effect on precipitin reactions of proteins or carbohydrates, such as horse serum or a precipitable substance of pneumococci, may be attributable to the more intricate constitution of the specifically reacting groups in these antigens.

Table III illustrates the inhibitory action of some cyclic acids on the precipitation by *o*- and *p*-aminobenzoic acid immune sera.

The tests show that not only benzoic acid but also other cyclic acids exert inhibiting effects. Intense inhibition was caused by α -thiophene carboxylic acid and α - and β -naphtoic acids, the result with the former being in good agreement with the close chemical similarity between α -thiophene carboxylic acid and benzoic acid.

Inhibition Reactions of Pronounced Specificity

The experiments reported in the present and a previous communication show that the reactions of certain immune sera can be inhibited by numerous substances, provided these are chemically somewhat similar to the reacting group of the antigen used. Such is the case when the groupings responsible for the specific precipitation have a simple structure, as for example in the antigens prepared with aminobenzoic acids. Extending the well known simile of E. Fischer, these instances may be compared to the unlocking of a simple lock by various keys or of various locks by a simple key. Accordingly it appears that the inhibitory effect can result from weak affinities which would not be sufficient for causing specific precipitation, *e.g.*, a serum for para-aminobenzoic acid would be inhibited to a certain degree by ortho-aminobenzoic acid but would not precipitate test antigens built up from the latter substance.

It has already been suggested that the range of reactivity is narrowed in cases in which either the antigen or the inhibiting substance is of somewhat more complex chemical structure. Inhibition reactions with azodyes (1) are instances of the sort. Since this view

has not been checked in any case by tests with numerous compounds of widely varied composition, and on account of the results reported in the preceding section, it seemed desirable to verify it by experiments carried out on a larger scale. To this end, two antigens and the corresponding immune sera, studied previously (12, 13), were selected with specific groups of more complicated constitution than those used in our first studies, namely two azoproteins, prepared from *l*-phenyl (para-aminobenzoylamino) acetic acid, and *l*-para-aminotartranilic acid. For the inhibition tests a large number of organic acids of widely varying chemical constitution were taken.

The following substances were used for the inhibition tests: (1) acetic acid; (2) normal butyric acid; (3) normal valeric acid; (4) isovaleric acid; (5) normal caproic acid; (6) isocaproic acid; (7) diethylacetic acid; (8) normal heptylic acid; (9) chloroacetic acid; (10) bromoacetic acid; (11) cyanacetic acid; (12) trichloroacetic acid; (13) β -iodopropionic acid; (14) lactic acid; (15) levulinic acid; (16) aminoisovaleric acid; (17) acrylic acid; (18) α -crotonic acid; (19) *d*, *l*-bromosuccinic acid; (20) citric acid; (21) fumaric acid; (22) glycocoll; (23) *d*, *l*-alanin; (24) phenylglycine; (25) *d*, *l*-phenylalanin; (26) *d*, *l*-leucine; (27) histidine; (28) benzoylglycine; (29) *d*, *l*-benzoylalanine; (30) *l*-asparaginic acid; (31) *l*-glutaminic acid; (32) camphoric acid; (33) cyclohexane carboxylic acid; (34) benzoic acid; (35) ortho-aminobenzoic acid; (36) meta-aminobenzoic acid; (37) para-aminobenzoic acid; (38) 4-chloro-3-aminobenzoic acid; (39) ortho-chlorobenzoic acid; (40) meta-chlorobenzoic acid; (41) para-chlorobenzoic acid; (42) ortho-bromobenzoic acid; (43) meta-bromobenzoic acid; (44) para-bromobenzoic acid; (45) ortho-nitrobenzoic acid; (46) meta-nitrobenzoic acid; (47) para-nitrobenzoic acid; (48) ortho-oxybenzoic acid; (49) meta-oxybenzoic acid; (50) para-oxybenzoic acid; (51) ortho-methylbenzoic acid; (52) meta-methylbenzoic acid; (53) para-methylbenzoic acid; (54) 2, 4, dinitrobenzoic acid; (55) 2, 4, 6, trinitrobenzoic acid; (56) anisic acid; (57) 1, 2, 5, dioxybenzoic acid; (58) 1, 2, 4, dioxybenzoic acid; (59) benzolsulfonic acid; (60) ortho-aminobenzolsulfonic acid; (61) meta-aminobenzolsulfonic acid; (62) para-aminobenzolsulfonic acid; (63) sulfosalicylic acid; (64) para-toluidinsulfonic acid; (65) para-azoxybenzoic acid; (66) phenylacetic acid; (67) mandelic acid; (68) benzilic acid; (69) vanillic acid; (70) cinnamic acid; (71) hydrocinnamic acid; (72) ortho-aminocinnamic acid; (73) meta-aminocinnamic acid; (74) para-aminocinnamic acid; (75) ortho-nitrocinnamic acid; (76) meta-nitrocinnamic acid; (77) para-nitrocinnamic acid; (78) ortho-cumaric acid; (79) phthalic acid; (80) isophthalic acid; (81) terephthalic acid; (82) α -thiophene carboxylic acid; (83) furan carboxylic acid; (84) meconic acid; (85) α -naphtoic acid; (86) β -naphtoic acid; (87) α -oxynaphtoic acid; (88) β -oxynaphtoic acid; (89) naphthionic acid; (90) α -pyrrole carboxylic acid; (91) picolinic acid; (92) nicotinic acid; (93) cinchonic acid.

For the tests 0.05 cc. of a neutral solution of the substance containing 0.5 millimol in 10 cc. water were used.

TABLE IV

Substance No.	Immune serum for levo-phenyl-(para-amino-benzoylamino) acetic acid		Immune serum for levo-para-amino-tartranilic acid		Substance No.	Immune serum for levo-phenyl-(para-amino-benzoylamino) acetic acid		Immune serum for levo-para-amino-tartranilic acid	
1	+	++	+±	++±	48	+	++	+±	++
2	+	++	+±	++±	49	+	++	+±	++±
3	+	++	+±	++	50	+	++	+±	++
4	+	++	+±	++±	51	+	++	+±	++
5	+	++	+±	++±	52	+	++	+±	++
6	+	++	+±	++±	53	+	++	+±	++±
7	+	++	+±	++	54	+	++	+±	++
8	+	++	+±	++±	55	+	+±	+±	++±
9	+	++	+±	++	56	+	++	+±	++
10	+	++	+±	++	57	+	++	+±	++
11	+	++	+±	++	58	+	++	+±	++
12	+	++	+±	++	59	+	++	+±	++±
13	+	++	+±	++±	60	+	++	+±	++
14	+	++	+±	++	61	+	++	+±	++
15	+	++	+±	++	62	+	++	+±	++
16	+	++	+±	++	63	+	++	+±	++±
17	+	++	+±	++±	64	+	++	+±	++
18	+	++	+±	++	65	+	++	+±	++
19	+	++	+±	++	66	±	++	+±	++
20	+	++	+	++	67	±	++	+	++
21	+	++	+±	++	68	±	++	+±	++
22	+	++	+±	++	69	+	++	+±	++±
23	+	++	+±	++	70	±	++	+±	++
24	±	++	+±	++	71	±	++	+±	++
25	+	++	+±	++	72	+	++	+±	++±
26	+	++	+±	++	73	+	++	+±	++±
27	+	++	+±	++	74	+	++	+±	++
28	±	++	+±	++	75	tr.	+	+±	++±
29	+	++	+±	++±	76	±	++	+±	++±
30	+	++	+±	++±	77	±	++	+±	++
31	+	++	+±	++±	78	+	++	+±	++
32	+	++	+±	++	79	+	++	+±	++±
33	±	++	+±	++±	80	+	++	+±	++±
34	+	++	+±	++±	81	+	++	+±	++
35	+	++	+±	++	82	±	++	+±	++±
36	+	++	+±	++	83	+	++	+±	++±
37	+	++	+±	++	84	+	++	+±	++±
38	+	++	+±	++	85	±	++	+±	++
39	+	++	+±	++±	86	±	+±	+±	++
40	+	++	+±	++	87	+	++	+±	++
41	+	++	+±	++±	88	+	++	+±	++
42	+	++	+±	++	89	+	++	+±	++
43	±	+±	+±	++±	90	+	++	+±	++
44	+	++	+±	++±	91	+	++	+	++
45	+	++	+±	++±	92	±	++	+±	++
46	+	++	+±	++	93	±	+±	+±	++
47	+	++	+±	++	Hom. subst.	0	±	0	0
					Control	+	++	+±	++

In the table, the first column gives the readings after 2 hours at room temperature for each of the two immune sera, the second column those after standing overnight in the ice box "Hom. subst." stands for "homologous substance," namely: levo-phenyl (para-aminobenzoylamino) acetic acid, or levo-para-amino-tartranilic acid.

It is seen from Table IV that in the tests with the first immune serum there occurred some inhibitions with non-homologous substances which, with one exception, disappeared or became very weak on longer standing. The homologous substance gave the strongest effect. With the immune serum for aminotartranilic acid only three heterologous substances showed relatively weak inhibition in the first reading, whereas the later reading demonstrated a perfectly specific reaction of the homologous substance. The inhibition with the homologous substance was complete also with one-half of the quantity used in the experiment presented and distinct with one-eighth of that quantity.

It may be concluded, therefore, that under appropriate conditions the inhibition tests exhibit a high degree of specificity, comparable to that of the common serological phenomena. Thus, the peculiar specificity displayed in serum reactions is not limited to high molecular colloids, but can be demonstrated as well with relatively simple crystallized substances, and is largely independent of the molecular size.

SUMMARY

Experiments are described which confirm the result that the specificity of inhibitory reactions involving substituted aromatic acids is decidedly influenced by the position of the substituent.

When antigens with specific groups of very simple constitution are used for the tests, inhibiting effects are obtained also with substances distantly related to those determining the reactivity of the antigens. On the other hand, if antigens are built up from protein and chemical compounds of somewhat higher complexity, the specificity of the inhibition reactions with synthetic crystallized substances is of the same order as that of the usual serum reactions; in other words, it is possible to distinguish such compounds by serological tests as readily as proteins can be differentiated with the aid of precipitating sera.

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TYPHUS FEVER

III. THE BEHAVIOR OF *RICKETTSIA PROWAZEKI* IN TISSUE CULTURES

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PLATE 28.

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INTRODUCTION

Cytological study of the scrotal sac exudate in typhus guinea pigs (Paper II of this series) suggested very strongly that *Rickettsia prowazeki* infects only mesothelial and endothelial cells. The study also brought out the fact that, *in vivo*, mesothelium appears to be a much more favorable medium for the growth of the organism than endothelium. Conclusive evidence was obtained that the organism does not multiply in phagocytic mononuclear cells (macrophages) and strong evidence against its multiplication in fibroblasts or epithelial cells. It therefore seemed worth while to restudy the problem of the propagation of typhus *Rickettsiae* in tissue cultures from the cytological point of view, using the technic already described (Paper II of this series) for the demonstration of the intracellular organism in sections.

Wolbach and Schlesinger (1) in 1923 set out tissue cultures, using infected brain tissue as explant material. They succeeded in maintaining virulence in their cultures for a maximum period of 28 days and they found *Rickettsiae* in sections of the culture as late as the 24th day. They probably obtained a pure culture of endothelium, since the neuroglia cells of the brain fragments did not multiply. They found *Rickettsiae* in their cultures only in relatively small numbers, which is not surprising in view of the scanty infection of their original tissue.

The scrotal sac exudate offers a much more favorable material from the point of view of the number of *Rickettsiae* originally present.

Fragments of this exudate which are barely visible to the naked eye are infectious, in the great majority of instances, after thorough washing in saline.

Zinsser and Batchelder (2) in 1930 set out cultures from this exudate and maintained virulence in their cultures for a period of 10 days. In 1929 and 1930 a large number of cultures were set out in this laboratory and we found that in spite of continued fairly good growth of cells, the cultures became non-virulent on or before the 16th day and usually before the 10th day. The cytological studies mentioned above suggested that this might be due to the fact that the mesothelial cells were outgrown by or transformed into fibroblasts. Maximow and others believe that the latter event invariably occurs.

Nigg and Landsteiner (3) recently reported that they were able, by using a modified Maitland medium, to maintain virulence *in vitro* for a period of 10 weeks. Their cultures were transferred every 8 days to fresh medium containing minced normal tunica.

The present tissue culture experiments are still in progress. The object of this preliminary report is to describe the appearance and location of the organisms in tissue cultures, and to depict certain interesting morphological changes which they undergo *in vitro*.

Technic

Explant Material.—The membranous exudate is removed from the surface of the testicle and floated in Tyrode's solution. The membrane, which is usually 0.1 to 0.2 mm. in thickness, is cut into fragments about 1 mm. square, by a rocking motion with a sharp curved knife.

Medium.—Blood is removed by heart puncture from a guinea pig and kept fluid during centrifugation by heparin in 1:10,000 dilution. Embryonic extract is prepared by grinding guinea pig embryos of 2 to 4 cm. in a sterile mortar, adding ten times the volume of Tyrode's solution to the pulp and centrifuging at high speed to get rid of the cells.

Cultures.—Several (6 to 8) drops of embryonic extract are placed in the bottom of a petri dish and a fragment of exudate membrane is picked up with a capillary pipette and floated in each drop. 1 drop of plasma is added to each drop of extract and before clotting each drop is stirred with the sealed end of a glass pipette. After clotting has occurred the petri dish is inverted and sealed by pouring a hot mixture of paraffin and vaseline into the space between the two sections. The cultures are usually incubated at 35–36°C. This temperature was adopted arbitrarily as being high enough to permit good growth of the cells and because it corresponds roughly to the temperature in the scrotal sac of the guinea pig, where

Rickettsiae multiply luxuriantly *in vivo*. Other temperatures are being used in experiments now in progress.

Transplants.—The vaseline-paraffin mixture is easily removed from the petri dishes with a stiff wire. The coagulated plasma drops are shaved off with a sharp scalpel and washed for 10 to 30 minutes in Tyrode's solution. They are then cut into two or four approximately equal fragments by a rocking motion with a curved knife and each fragment is reembedded in a fresh drop of plasma and embryonic extract. The cultures are usually transplanted every 2 to 4 days, depending on the rapidity of the growth and evidence of beginning liquefaction of the plasma.

On each transfer day some of the cultures in each group were injected into a guinea pig, some were transferred and some were fixed and stained by the method previously described (Paper II of this series).

Description of Cultures

During the first 12 to 24 hours a grossly visible greyish white "halo" appears around the fragments of exudate. Microscopically the cells are largely isolated ameboid cells, many of which have numerous cytoplasmic processes so that they resemble ganglion cells from the central nervous system. A variable number of polymorphonuclears also wander out from the exudate at this stage. During the next 48 hours the picture changes markedly. The isolated cells decrease in number and cells of a very different appearance grow out from the surface of the original fragment. These cells first appear as sprouts of six to twelve or more cells joined end to end, but later they form a solid mass about the original fragment. These cells have pale, almost invisible nuclei, and frequently show fine fat droplet vacuolization of their cytoplasm, so that the nucleus appears as an oval, apparently empty, space in the more refractile vacuolated cytoplasm. They resemble epithelium in their manner of growth and never branch and ramify like typical connective tissue cells. They do not phagocytose particles of graphite ink added to the cultures. *Rickettsiae* grow luxuriantly in these cells and we believe that they are mesothelial cells (See Paper II of this series) although we have not been able to distinguish them with certainty from fibroblasts.

In successive transfers, the ameboid phagocytic cells almost entirely disappear and the probable mesothelial cells appear to be left in pure culture. The growth energy of these cells appears to diminish, however, and they multiply very slowly after the fourth or fifth transfer.

Topography of Rickettsia in Sections of Tissue Cultures

Thus far twenty-eight first generation cultures of scrotal sac exudate (of 3 to 6 days' duration) have been studied histologically. *Rickettsiae* were present in large numbers in all. The material for these twenty-eight individual cultures came from six different guinea pigs, two of which were killed on the 1st day of obvious scrotal swelling, one on the 2nd day and three on the 3rd day. Smears of the exudate in three cases showed numerous *Rickettsiae*, both intracellular and extracellular, in one case moderate numbers of extracellular forms and a rare infected cell, and in one case only a very few extra-

TABLE I
Culture 5, Set Out February 10, 1931

Date of fixation	Growth	Infected cells	Guinea pig inoculations
Feb. 14.....	Excellent	Very numerous	Positive
Feb. 19.....	Fair	Few (1 or 2 per section)	Negative*
Feb. 23.....	Fair	None found*	Positive

* The positive guinea pig inoculation on Feb. 23, when no *Rickettsiae* were found in the sections, probably indicates either that the particular culture chosen from the group for microscopic study was sterile or that *Rickettsiae* were present in such small numbers that they were not found. The negative guinea pig inoculation on Feb. 19 is explainable in a similar manner.

cellular¹ forms after long search. The cultures set out from the exudate in which only a few *Rickettsiae* were demonstrated by smear happened to be the most heavily infected of all.

First generation cultures (of 3 to 6 days' duration) practically always contained infected cells in every section and often as many as 40 infected cells in a single section. Several groups of cultures have been sectioned in the second and third generations. In the majority of instances, the *Rickettsiae* disappeared quite rapidly in successive transfers. The example cited in Table I is typical of these results.

¹ The extracellular forms are undoubtedly spilled from cells in making the smear (see Paper II of this series).

In one group of cultures, however, to which fragments of striated muscle had been added, *Rickettsiae* were found in very great numbers in the fourth and fifth generations after 16 and 21 days *in vitro*. The polymorphonuclears had entirely disappeared by this time and the large pale, vacuolated cells (believed to be mesothelial cells) were enormously distended with organisms. In one half of the 21 day culture practically every cell was very heavily infected. This culture showed the heaviest infection of all, in spite of the fact that the growth of the cells was scanty and had apparently ceased several days before the culture was fixed. In this instance the organisms appeared to have multiplied extensively in the cytoplasm of degenerating cells.

Rickettsiae when found were always within the cells except in one or two instances where cells had been torn by the knife and a few clusters of organisms had been carried for a short distance away from the cell in which they belonged.

Rickettsiae were found in growing cells along the edge of the fragments and in smaller numbers in cells which had grown outward from the fragment in the form of long strands. In the interior of the fragment certain cells survive and grow in the form of a sponge-like network, enclosing small areas of dead tissue. Mitotic figures were nearly as numerous in the central part of the tissue as at the periphery and *Rickettsiae*-filled cells were often found in the interior.

The *Rickettsiae*-laden cells usually had a definitely focal distribution. Frequently the infected cells in a given section were confined to an area representing only a small portion of the entire section. In other sections groups of five or six adjacent cells all showed infection. In several instances *Rickettsiae* were found in large numbers in cells which were undergoing mitotic division.

The cultures contained two definite types of cell (exclusive of the leucocytes). The appearance of these before fixation has been described above. The *Rickettsiae* were always found in the large non-ameboid, non-phagocytic cells which are regarded as of probable mesothelial origin. The ameboid macrophages, which were recognized by their content of hemosiderin or by the fact that they had taken up graphite ink, never contained *Rickettsiae*. The cells in which the *Rickettsiae* grow have large pale oval or rounded blue staining nuclei with several nucleoli and very abundant finely vacuolated cytoplasm, the reticulum of which stains pale blue. (The *Rickettsiae* are stained

a deep purplish red.) These cells grow in sheets and strands, and often appear to have well defined cuticular borders (Fig. 8).

Morphology of the Organisms

The organisms showed a wide range of morphological variation. In some cultures they appeared in the form of diplobacilli, similar to those seen in smears and sections of the scrotal sac exudate. In other cultures they appeared as long threads, ranging up to 20 microns in length. In still other cultures both forms of the organism were present and occasionally both forms were found in the same cell with various transitional stages. The morphology of the organism did not seem to be in any way correlated with the age of the culture or with the rapidity of growth of the cells but the thread forms were most often found in lightly infected cells at the growing edge of the cultures.

Many of the threads appeared solid but in the preparations where the fixation and staining appeared most perfect they were composed of long chains of diplobacilli and we believe that they are always formed in this way. In many instances the thread forms appeared to branch but we believe that true branching does not occur.

The variation in size is extraordinary. Minute granular forms on the borderline of visibility (Fig. 5) were often found in the cells which were distended with organisms, while the larger forms were usually seen in cells which were only lightly infected (Fig. 8). The smallest forms appear as minute single or paired cocci but the presence of transitional forms suggests that they are probably small plump bacilli. These forms are so minute that it is impossible to determine their exact shape.

The organisms were always stained a uniform deep purple and never appeared as diphtheroids. A few cells in the cultures of longest duration contained irregularly swollen very deeply staining structures similar to involution forms. In a few instances the *Rickettsiae* appeared to be breaking up and degenerating in the cytoplasm of cells which appeared to be healthy.

COMMENT

The presence of organisms in cells which have grown away from the original fragment indicates that new cells become infected after the

culture is set out. The presence of organisms in dividing cells proves that one method of spread of the infection is by hereditary transmission. This mechanism would also explain the focal nature of the infection. These studies do not indicate whether or not the infection spreads directly from cell to cell by contact, *via* intercellular bridges, or *via* the plasma clot. In the majority of our cultures the infection appears to spread very slowly. The extremely heavy infection found in the 21 day culture, however, suggests that under certain conditions the infection may spread rapidly and involve all cells present in a large area. Whether immunity to the organism may be inherited is still an unanswered question.

The absence of *Rickettsiae* from macrophages corroborates the conclusion which we formed from a study of the cytology of the exudate *in vivo*.

The organism appears much more pleomorphic in tissue cultures than *in vivo*, and the range of morphological variation (see illustrations), corresponds closely to that described in the louse. Wolbach (3) believed that the thread forms represented the early stages of intracellular multiplication and this concept is somewhat borne out by the fact that these forms were most often seen in lightly infected cells at the growing edge of the culture. No observations were made in this study which indicate that granules within the large thread forms are extruded and become viable as has been suggested (4).

The improved method of fixation and staining of *Rickettsiae* seems to afford a valuable method for further study of the behavior of the organisms when brought in contact with cells of known types and the effects of variations in temperature, pH, gas tensions and composition of the medium on the number and morphology of the *Rickettsiae* found in cultures.

SUMMARY

Typhus *Rickettsiae* are found in large numbers in sections of tissue cultures of scrotal sac exudate.

Extensive multiplication of the organisms occurs, and new cells become infected. Organisms are seen in cells undergoing mitotic division.

The organisms usually become less numerous after the 6th day in

vitro, but in one instance organisms were extremely numerous on the 16th and 21st days.

Rickettsiae in tissue cultures retain their intracellular location, even when infection is very heavy.

They multiply exclusively in non-phagocytic cells which are believed to be of mesothelial origin.

Pleomorphism is much more pronounced in tissue cultures than in guinea pig tissues and is entirely comparable to that seen in the louse.

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EXPLANATION OF PLATE 28

All illustrations are camera lucida drawings from paraffin sections of tissue cultures. For technic of fixation and staining, see Paper II of this series. The drawings were all made at a constant magnification of 1250 diameters.

FIG. 1. A group of cells from a 6 day culture. One of the three *Rickettsiae*-laden cells is in mitosis.

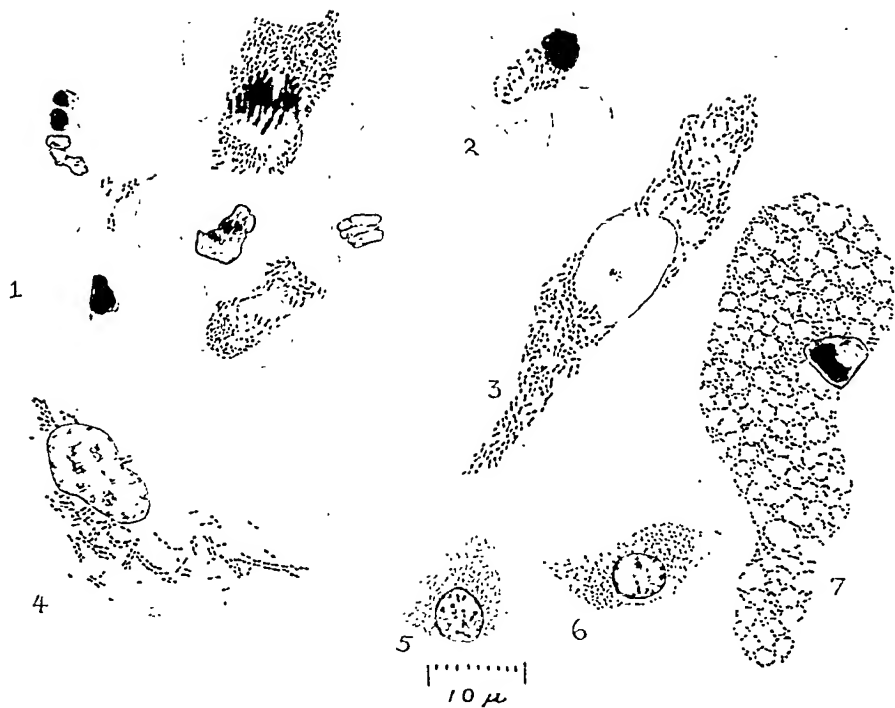
FIG. 2. An infected cell from an 8 day culture.

FIGS. 3 and 4. Cells infected with large bacilliform *Rickettsiae*. From a 4 day culture.

FIGS. 5 and 6. Cells infected with the minute coccoid type of *Rickettsia*. From a 4 day culture.

FIG. 7. A large degenerating cell from a 5th generation culture of 21 days' duration. Note the arrangement of the organisms around the fat vacuoles. Practically every cell in several low power fields was heavily infected in this manner.

FIG. 8. Large thread forms (chains of diplobacilli) in an outward growing strand of mesothelial (?) cells. First generation culture of 4 days' duration.



DIFFERENTIATION BETWEEN CERTAIN TOXIC PROPERTIES OF FILTRATES OF HEMOLYTIC STAPHYLOCOCCUS AUREUS

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That sterile filtrates of hemolytic staphylococci are toxic for the cells of the body has been shown by their destructive action (1) on leucocytes (the leucocidins (1-4)), (2) on erythrocytes (the hemotoxins (5-8)), (3) on the skin (the dermatoxins or necrotoxins (9-14)), and (4) by their quick killing effect when injected intravenously in rabbits ("acute killing poison" (15-17)).

The evidence, however, is contradictory as to whether these various toxic effects are due to one or several distinct substances. The view that they are due to the same substance is based upon the following observations: (a) The poisons are all exotoxins with roughly the same heat stability. (b) The relative proportions of the different toxins in a toxic filtrate are approximately the same. (c) The antibodies produced by various methods against the live organisms or against the toxic filtrates, show equivalent neutralizing activities against the various toxic actions (hemotoxic, necrotoxic, or quick killing actions).

Not all observers are willing to accept the idea that the toxic actions described are due to one and the same substance. For instance, several observers have not been able to demonstrate the same relative proportion of the different poisons in the toxic filtrates; while Neisser and Wecksberg (4), using leucocytes, were able to adsorb the leucocidin but not the hemotoxin from the filtrates, though they do not give the protocols of their experiments.

In previous papers (12, 13), one of us has reported the results of our studies of the necrotoxin in staphylococcus filtrates. Although in those papers no experiments were described which bear directly on the question of the unity of the necrotoxin and hemotoxin, our impression was that the necrotoxic and hemotoxic effects were due to separate constituents in the filtrates, because many of the cultures investigated were extremely hemolytic when streaked on blood plates and yet produced no demonstrable necrotoxin by the method used. At that time very few attempts had been made to study the killing properties of the toxic filtrates when given intravenously. However, we had observed that two young rabbits (weights 1030 and 710 gm.) died 8 hours after receiving intravenous injections of 2 cc. of the necrotizing poison, whereas large rabbits were insusceptible to it, even in large

doses. Later, we obtained several filtrates which killed large rabbits (2000 gm.) in less than 24 hours in small amounts: *viz.*, 0.1 to 0.2 cc., but these filtrates lost their toxicity after standing in the ice box for 7 days. No work was done to determine if a quantitative relationship existed between the killing, the necrotoxic, or the hemotoxic effects in these latter filtrates.

Recently Burnet (14) has studied the relationship which the necrotoxin, hemotoxin, and killing poisons of the staphylococcus bear to one another. He found that there was a quantitative relationship between the killing and necrotizing effects and a fairly quantitative relationship between the hemotoxic and killing effects in all filtrates from the three strains studied; and that in five antitoxins which had been produced by different methods, all showed a constant relationship in antitoxin for the three toxic activities. Based on these observations, he concludes that these three toxic activities are all manifestations of a single antigenic substance; in other words, that the staphylococcus produces a single exotoxin which destroys erythrocytes, skin, and capillary endothelium.

We wish to report some experiments which seem to establish the fact that there are at least two, and probably more, antigenically distinct exotoxins in toxic filtrates of the *Staphylococcus aureus*. The experiments to be described may be divided into two groups; first, those dealing with the relative proportions of hemotoxin, necrotoxin, leucocidin, and the "acute killing poison" in filtrates of hemolytic *Staphylococcus aureus* cultures; and second, those dealing with the selective adsorption of the toxins from the filtrate by erythrocyte stroma or by leucocytes.

EXPERIMENTAL

Preparation of Media.—A modification of Walbum's (18) medium was used throughout this work. This consisted of a sugar-free veal infusion broth to which 1 per cent Witte peptone, 1 per cent Difco peptone, 0.2 per cent KH_2PO_4 , and 0.03 per cent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were added.

*Sources of Cultures.*¹—Four strains of the hemolytic *Staphylococcus aureus* were used. These strains were virulent for rabbits, death occurring within 1 to 3 days after receiving intravenous inoculations of 0.05 to 0.2 cc. of a broth culture.

The virulence of a strain was found to be roughly proportionate to the quantity

¹ The sources of the strains used were as follows:

779. 7/10/30 from a pustule obtained from Dr. Galbreath in Porto Rico.

814. 12/1/30 from a blood culture obtained from Dr. McKinley in Porto Rico.

805. 12/1/30 from a blood culture from a case of acute endocarditis at the Presbyterian Hospital.

782. 12/17/30 from a skin lesion from the Vanderbilt Clinic.

of hemotoxin it produced. Since our previous work indicated that the virulence of a strain disappeared rapidly when subcultured on agar, in these experiments inoculations of the broth for toxin production were made only from the original blood agar slants.

Growth of the Staphylococcus.—The staphylococci were grown aerobically in Erlenmeyer flasks two-thirds full of the medium. Growth was allowed to continue until the pH, which always dropped to 6.4 to 6.8 for the first few days of growth, had risen to 7. This required from 10 to 16 days, depending on the strain and batch of medium used. After incubation, the cultures were distributed in tubes, chilled, vaseline seals added, and centrifuged. The clear chilled supernatant fluids were then passed through Berkefeld V filters. The filtrates were preserved in the ice box under vaseline seals. With these precautions to prevent oxidation, the filtrates retained all their toxic properties for at least 4 months.

For testing the action of the filtrates to kill rapidly, young rabbits weighing between 700 and 1000 gm. were used. The reaction of the small rabbits to the same dose of filtrate is remarkably regular. For skin tests, rabbits weighing from 1500 to 2000 gm. were used. From eight to sixteen tests with various dilutions of toxins may be inoculated into one animal.

Hemotoxin Tests.—Tests for hemotoxin were carried out in the usual way. One unit of hemotoxin was taken as the smallest amount of a filtrate which completely hemolyzed 2 cc. of 1 per cent well washed rabbit red cells in 40 minutes.

Necrotoxin Tests.—One unit of necrotoxin was taken as the smallest amount of filtrate or diluted filtrate which, when injected intradermally, will cause definite necrosis of an area of 5 mm. in diameter.

Leucocidin Tests.—The leucocyte suspensions for the leucocidin tests were obtained from rabbits following the intrapleural injections of aleuronat. 18 hours after injection, the animals were bled to death from the carotid, and the pleural exudates taken up in one-third their volume of 0.5 per cent sodium chloride solution containing 2 per cent sodium citrate. Only those exudates showing no reddish color and containing very few, if any, red cells, were used. The exudates were then titrated to determine the proper amount to use in the leucocidin tests. For this purpose, 0.05 cc. of 1:10,000 solution of methylene blue was added to varying amounts to the leucocyte suspension in precipitin tubes, and the volume in each tube brought up to 1 cc. with saline solution. Vaseline seals were added to all the tubes, and they were put in a water bath at 37°C. The volume of the leucocyte suspension used in the leucocidin tests was the amount that would reduce the methylene blue completely in 20 minutes.

The leucocidin tests were set up as follows: the previously determined quantity of the leucocyte suspension was added to various amounts of the staphylococcus filtrate to be titrated, and the volume of all tubes brought up to 1 cc. with saline. One control tube containing the largest dose of the filtrate used in the experiment was heated at 60°C. for 20 minutes, and a second contained the leucocytes without the filtrate. All tubes were placed in the water bath at 37°C. for 1 hour, then 0.05 cc. of 1:20,000 of methylene blue and a vaseline seal were added to each; and

finally they were reincubated for 1 hour. One unit of leucocidin represented the minimum amount of a filtrate which completely prevented the reduction of methylene blue by the leucocytes in 1 hour.

"Acute Killing" Tests.—One unit of the "acute killing" toxin represented the smallest amount of filtrate which, when injected intravenously, would kill a 700 to 1000 gm. rabbit within 1 hour. The symptoms of rabbits which die soon after the intravenous injection of staphylococcus filtrates have been described by others. For some time after receiving the injection the animals appear normal, but then become unsteady, fall over, breathing becomes rapid and shallow, and they become progressively weaker until death, which usually occurs in less than 1 hour after injection. At autopsy nothing abnormal is to be seen. If the dose of toxin is so adjusted that the rabbits survive for 12 to 48 hours, a very different picture presents itself. 1 to 3 hours after injection they flatten out on their abdomens, appearing weak, and they become progressively weaker till death.

At autopsy marked changes are always present in the kidneys. In the animals which die in 12 to 24 hours, the kidneys are mottled deep red against a pale background or are a deep purplish red throughout. In those which survive longer, *viz.*, 24 to 48 hours, the kidneys are a deep red, mottled with yellow areas varying from 1 to 10 mm. in diameter. The histological changes in these kidneys will be taken up in a later publication.

A. Experiments Dealing with the Relative Proportions of the Various Toxins in the Staphylococcus Filtrates.—Our first experiments were devised to determine the accuracy of Burnet's claim that the three toxic activities—(hemotoxic, necrotoxic, or "acute killing")—bear constant ratios to one another. A large number of filtrates from our four toxic strains were titrated for these three toxins, some also being tested for leucocidins.

Our results may be briefly summarized as follows: In general, filtrates which were strong in one toxic activity were strong also in the others. In the strongly hemolytic filtrates, *viz.*, those containing 100 to 200 hemotoxic units per cubic centimeter, there appeared to be a fairly definite ratio between the hemotoxic, necrotoxic, and "acute killing" activities. This same quantitative relationship held for all strong hemotoxic filtrates from the four strains studied. Roughly, one "acute killing" unit was equivalent to 25 hemotoxic and to 80 to 100 necrotoxic units. In filtrates with less hemotoxic strength this quantitative relationship did not hold, especially as regards the ratio of the hemotoxic to the necrotoxic units in a filtrate. There appeared to be no definite relationship between the leucocidins in a

filtrate and any other toxic properties. The results of these experiments are summarized in Table I.

It is clear that no definite conclusions as to the unity or plurality of poisons in staphylococcus filtrates can be drawn from these experiments. More convincing results were obtained in the following experiments.

B. Experiments Dealing with the Adsorption of Staphylococcus Toxic Filtrates with Erythrocyte Stroma or with Leucocytes.—In a previous paper, it was proved conclusively by adsorption of pneumococcus toxic autolysates with red cells in the cold, that the necrotoxin and the hemotoxin of the pneumococcus are separate entities. On analogy, one would suppose that the staphylococcus hemotoxin and necrotoxin are likewise different substances.

Neisser and Wecksberg (4) were able to remove staphylococcus hemotoxin from the toxic filtrates by treatment with red blood cells

TABLE I
Units of Toxins Equivalent to One Killing Unit

Filtrates	Killing dose	"Acute killing" unit	Hemotoxins	Necrotoxins	Leucocidins
Strongly toxic.....	0.1 to 0.25 cc.	1	25	80-100	5-20
Weakly toxic.....	0.5 to 2 cc.	1	40-60	80-300	5-20

in the cold. We have made many attempts to confirm their findings, both with sheep and rabbit red cells, but always with negative results. It appeared that under the conditions of our experiments, staphylococcus hemotoxin and red cells combined only at higher temperatures, where hemolysis also occurred and obscured the results.

We next attempted to bind the hemotoxin with erythrocyte stroma, a procedure which could be carried out at 37°C. By this method, we were successful in obtaining almost complete selective adsorption of the hemotoxin with either rabbit or sheep red cell stroma, leaving the necrotoxin undiminished in the filtrate. When the stroma-adsorbed filtrates were tested for the presence of leucocidin it was found that most of this toxin had also been removed from the treated filtrates. Very little of the leucocidin could have been removed by the relatively small amounts of leucocytic substances present in the stroma prepara-

tions, for in our later experiments in which we used leucocyte suspensions as the adsorbing agent, very thick leucocyte suspensions were required to remove this toxin. As noted before, Neisser and Wecksberg also found that red cells adsorbed leucocidin as well as hemotoxin from staphylococcus filtrates. Because of insufficient material, only two young rabbits, weighing 610 and 835 gm., were injected intravenously with 1 and 0.75 cc. respectively of the stroma-adsorbed filtrates, and both survived. These rabbits were killed 3 days after receiving the injections and were found to have no lesions in the kidneys. The untreated filtrate diluted one-half killed rabbits in 0.2 and 0.3 cc. amounts respectively. The survival of these two rabbits and the absence of kidney lesions at autopsy, appear to indicate that the substances responsible for sudden death and the kidney lesions had been removed from the toxic filtrates by the red cell stroma

TABLE II

Amount in Cubic Centimeters Containing One Unit of the Various Poisons

Filtrate	Hemotoxin	Necrotoxin	Leucocidin
Stroma treated.....	0.15	0.0035	0
Untreated.....	0.015	0.0035	0.05

and therefore cannot have been associated with the necrotoxic action of the filtrates. A typical protocol of an experiment with a stroma-treated filtrate is given below:

Experiment.—To 1 cc. of packed stromata obtained from 50 cc. of rabbit red cells, was added 2 cc. of a staphylococcus filtrate plus 2 cc. of normal salt solution, and the preparations well mixed. The control tube contained 2 cc. of the same filtrate plus 2 cc. of normal salt solution. After adding paraffin oil to both tubes, they were placed in the water bath at 37°C. for 2 hours and then in the ice box overnight. The following morning the tube containing the stroma was centrifuged and the clear, slightly reddish supernatant fluid tested for the various toxic effects along with the control. While being centrifuged, the tube was kept immersed in ice water.

The results are summarized in Table II.

The experiment recorded in Table II seems to show that the red cell shadows have removed nine-tenths of the hemotoxin and the leucocidin, leaving the necrotoxin undiminished in the filtrate.

The purpose of our next experiments was to find out if the leucocytes selectively adsorb one or more of the toxins from a toxic filtrate. In their work on this point, Neisser and Wecksberg (4) state that in the cold, leucocytes remove the leucocidin and not the hemotoxin from staphylococcus toxic filtrates.

The results of our experiments along this line have not been conclusive and therefore will not be given in detail. We found that the leucocytes sometimes did, and sometimes did not, remove the hemotoxin from the filtrates. Only very thick suspensions of leucocytes without a trace of red cells were used, so that the removal of the hemotoxin could not have been due to the presence of red cells in the preparation. However, in contrast to this, the leucocidin was invariably almost completely removed by the leucocytes; whereas the necrotoxin remained undiminished in the leucocyte-treated filtrates. Taken as a whole, these last experiments with leucocyte-treated filtrates bring out nothing new but confirm the points brought out in the red cell stroma experiments; *viz.*: that the necrotoxin is a different toxic principle from either the hemotoxin or the leucocidin.

CONCLUSIONS

1. Sterile filtrates from certain hemotoxic strains of *Staphylococcus aureus* have several toxic properties, of which the most important are the hemotoxic, the necrotoxic, the leucocidic and the property of killing rapidly.
2. The necrotoxic action appears to be caused by a constituent in the filtrates different from either the hemotoxic or the leucocidic one.

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THE GROWTH AND DEATH OF TISSUE CULTURES EXPOSED TO SUPRANORMAL TEMPERATURES

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PLATE 29

(Received for publication, June 1, 1931)

In an attempt to investigate the nature of the recovery of tissues from sublethal exposures to supranormal temperatures it was noted that cultures exposed to higher temperatures for short periods of time and then returned to an incubator at 39°C. would grow at essentially the same rate as controls kept continuously at 39°C. Exposures for longer intervals of time led to an inhibition of growth, and it was hoped that by employing various intervals of exposure at various temperatures some measure of this inhibition would be had. It is a matter of some interest also that beyond a certain critical exposure time *all* growth is inhibited and death ensues, and we have been interested to ascertain whether the lethal exposure represents a period in which all cells are killed directly or whether some factor necessary for their growth is destroyed. The departure of one of us from Germany before all the experiments could be completed prevented a complete answer to the problems we set ourselves. At the same time we believe that certain interesting implications in the available data are worthy of mention.

Method

The experimental material consisted of pure cultures of chick osteoblasts. The tissue was cultured ordinarily in Carrel flasks containing chicken blood plasma and a supernatant fluid consisting of 15 per cent embryonic juice in Tyrode solution. The supernatant fluid was changed every 3 days (see Fischer, 1930). Certain cultures were carried on in the ordinary way on cover-glasses, the medium consisting usually of equal parts of 25 per cent embryonic tissue juice and blood plasma. The ensuing account will be confined almost exclusively to the behavior of tissues grown in Carrel flasks.

The cultures were prepared by cutting the tissue of a healthy, growing culture of osteoblasts into two approximately equal sized pieces. One of the transplants, used as a control, was placed in a flask that was kept in an incubator at 39°C. The other transplant was exposed to the higher temperature by placing the flask in a water bath, and then returned to an incubator at 39°C. after a definite interval. Records of the growth of the cultures were made by daily measurements of their areas. Measurements were taken from 3 to 8 days after the initial transplantation. The inaccuracies ordinarily attributed to areal measurements are apparently of no great significance in our experiments, since both growth inhibition and death are very definitely indicated in the growth curves obtained.

Experimental cultures were exposed to five temperatures ordinarily considered supranormal, namely: 42°C., 44°C., 47°C., 50°C., and 52°C. In all the experiments to be described the temperature never varied by more than +0.1°C. during exposure periods, and usually the variation was much less. In order to insure rapid and effective thermal penetration the flasks were immersed in the water of the thermostat, except for certain experiments conducted at 42°C. where prolonged exposures necessitated placing the cultures in an air chamber that was in turn immersed in a properly heated water bath.

Our first, rather surprising, experience was that cultures growing at 42°C. would, apparently, continue to do so for an indefinite period. No lethal exposure period could be found. Furthermore, the growth of cultures at 42°C. continued at approximately the same rate as in the control cultures at 39°C. The only indications of any growth inhibition occurred in the so called "faster growing cultures" exposed for fairly long periods (Chart 1).

It will be seen that all cultures exposed to 42°C. for more than 30 hours continue to grow, but at a relatively slow rate, so that after 9 days of growth they attain an area of approximately 80 units. In fact the growth rates of cultures exposed to 42°C. for more than 30 hours are practically identical whether the exposure has been for 45 hours or 94½ hours, and no matter what rate of growth the control cultures exhibit. It is as though exposure to 42°C. beyond a certain time results in the induction of a definite slow growth rate. We have not exposed cultures to 42°C. for more than 130 hours and it is possible that extremely lengthy exposures might result in lethal effects.

Cultures exposed to 44°C. also proved exceptionally resistant. Certain cultures kept at this temperature for 124 hours continued to live and grow. In an attempt to discover if any lethal effect could be had a set of five cover-slip cultures was prepared. These were kept

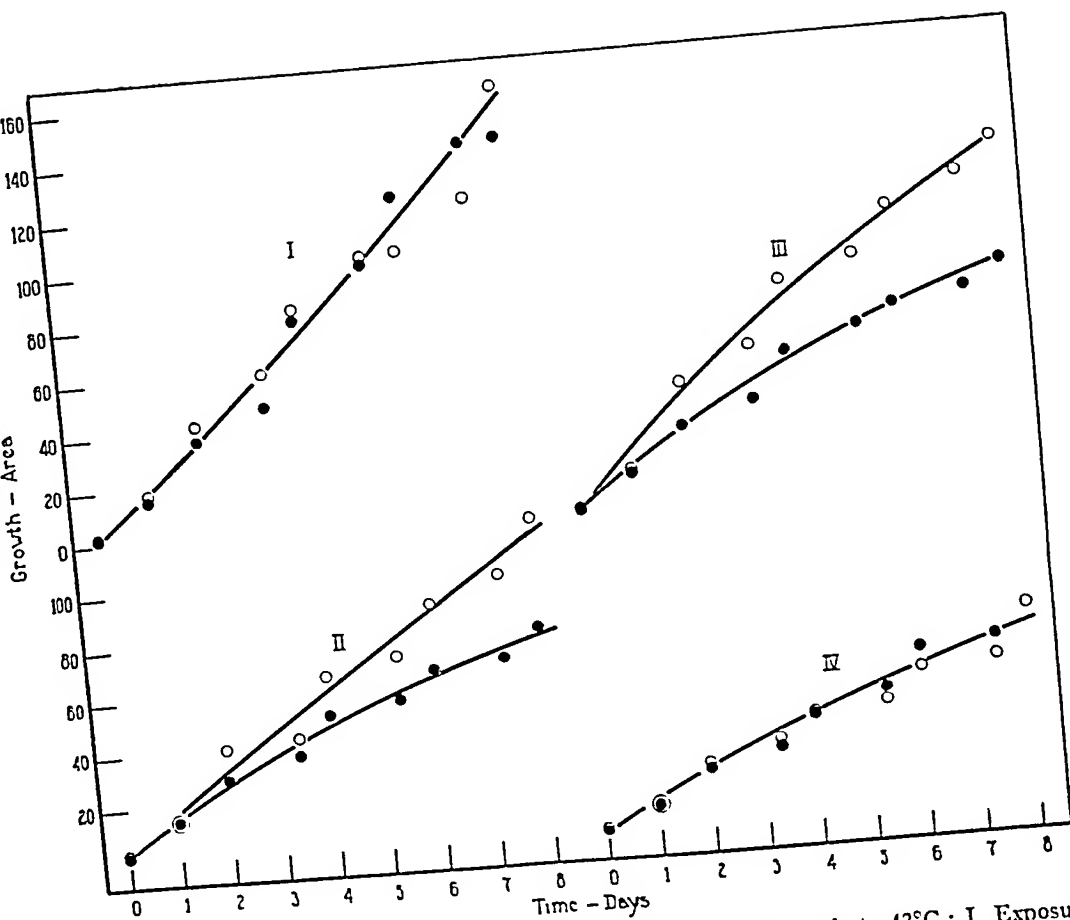


CHART 1. Growth of cultures in Carrel flasks exposed for varying intervals to 42°C.: I. Exposure time = 30 hours; II. Exposure time = 45 hours; III. Exposure time = 54 hours; IV. Exposure time = 90 hours. In this and all succeeding figures open circles represent data for the control cultures at 39°C.; closed circles represent data for the experimental cultures. Note that for all exposures beyond 30 hours the experimental cultures grow at approximately the same rate regardless of the exposure time or the growth rate of the control cultures.

at 44°C. and transplanted to fresh media every 2 days. After 144 hours one culture was still alive and four were dead. In another series one such culture showed the growth of a few cells after 168 hours exposure to 44°C.

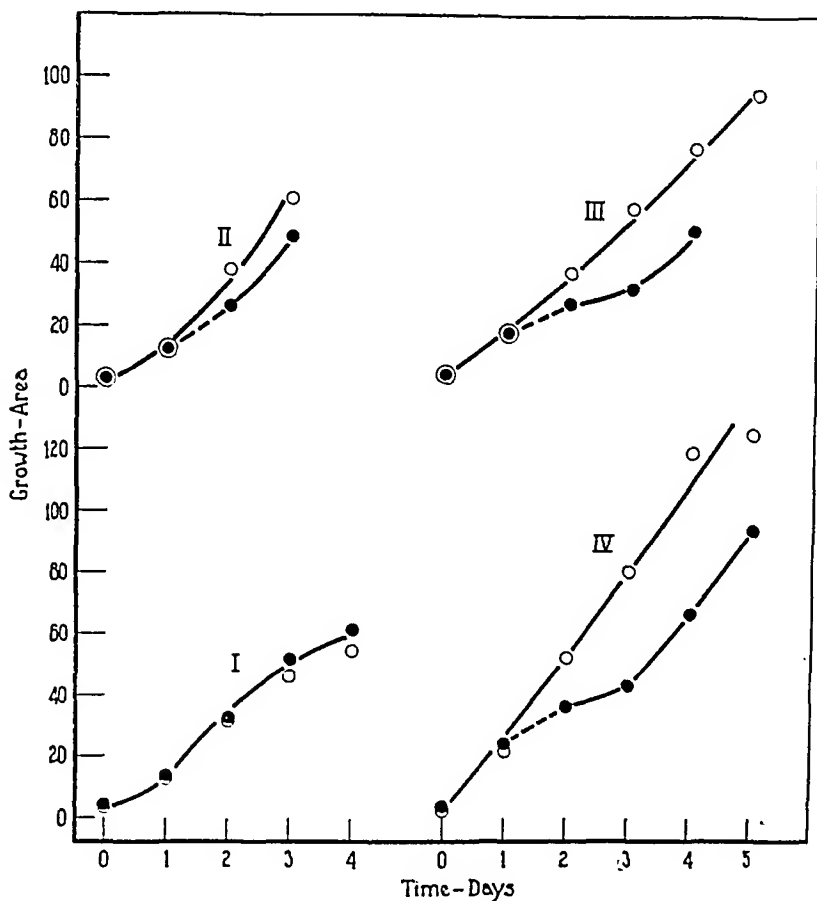


CHART 2. Growth of cultures exposed for varying intervals to 44°C.: I. Exposure time = 1½ hours; II. Exposure time = 3 hours; III. Exposure time = 15 hours; IV. Exposure time = 46 hours.

Despite the fact that no lethal exposure time was observed in our flask cultures, a very obvious inhibition of growth occurred in all exposures lasting 3 hours or more. This is graphically illustrated in Chart 2. The growth inhibition does not manifest itself until after an appreciable latent period; then, as the curves illustrate, the growth rate falls off even though the cultures have been returned to the incu-

bator at 39°C. before any effect on the growth rate is noticeable. This latent period preceding the change in growth, curiously enough, appears to be about 1 day in length whether exposures have been made for 3 hours or for 46 hours. Since our measurements were made every 24 hours it is difficult to decide exactly the length of this latent period, but it is certainly between 24 and 48 hours long in each case. We had

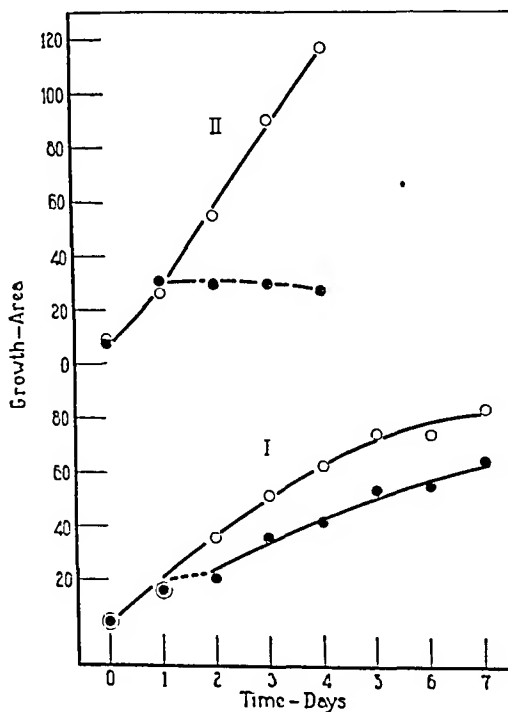


CHART 3. Growth of cultures exposed for varying intervals to 47°C.: I. Exposure time = 40 minutes; II. Exposure time = 60 minutes.

hoped to obtain indications of the reduction of this latent period with increasing periods of exposure, but no such indications are to be had, at least within the limits of determination possible with our methods of measurement.

One other consideration worthy of mention here is that cultures exposed to 44°C. for 3 hours or longer will show this definite inhibition of growth, although after a period of time the growth rate will again become almost normal.

When exposures were made at 47°C. definite lethal effects were observed. Cultures exposed to 47°C. for 1½ hours always die. Cultures exposed for 1 hour will in some instances die, in others live. Although cultures exposed for less than an hour invariably continue to grow there is a definite inhibition of growth in cultures exposed for

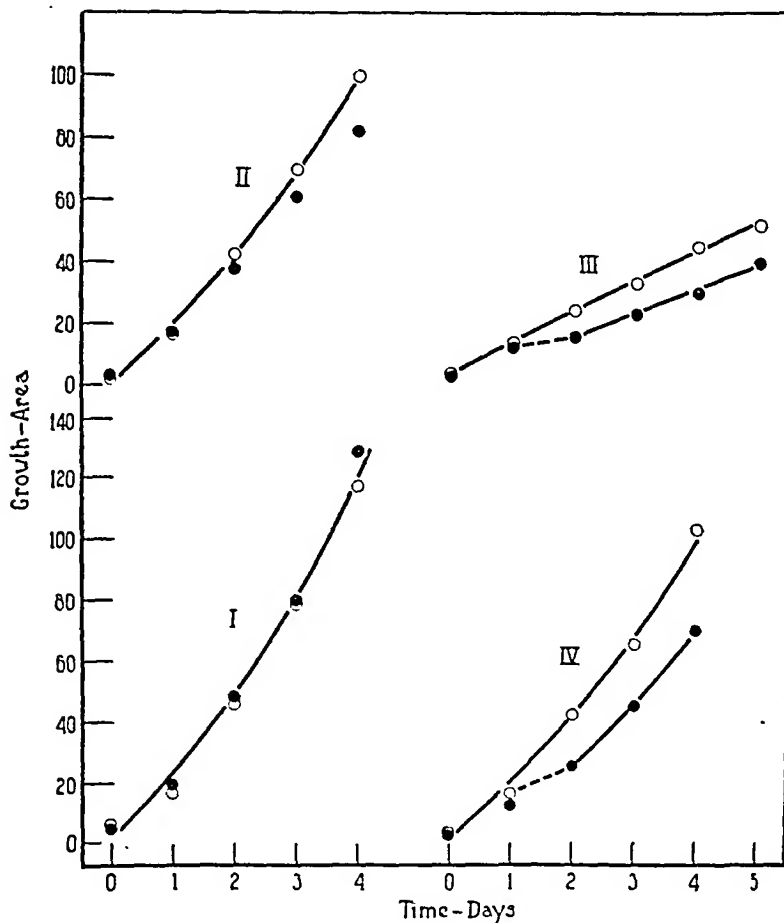


CHART 4. Growth of cultures exposed for varying intervals to 50°C.: I. Exposure time = 1 minute; II. Exposure time = 2 minutes; III. Exposure time = 3 minutes; IV. Exposure time = 4 minutes.

30 minutes. An exposure of less than 30 minutes results in no detectable inhibition of growth.

Here again the latent period preceding definite growth inhibition is approximately 24 hours, irrespective of the length of exposure. Similarly, in cultures which have been subjected to lethal exposures there

is a definite growth for 24 hours and then all growth ceases and death ensues. This is illustrated graphically in Chart 3 where the data are presented for a culture dying after 1 hour's exposure to 47°C. Note that apparently for 24 hours the experimental culture continued to grow at the same rate as its control, or at a slightly faster rate. Observe also that this 24 hour latent period preceding any manifestation of the effect of exposure is similarly exhibited in a culture receiving a sublethal exposure. In this and in all succeeding experiments we have

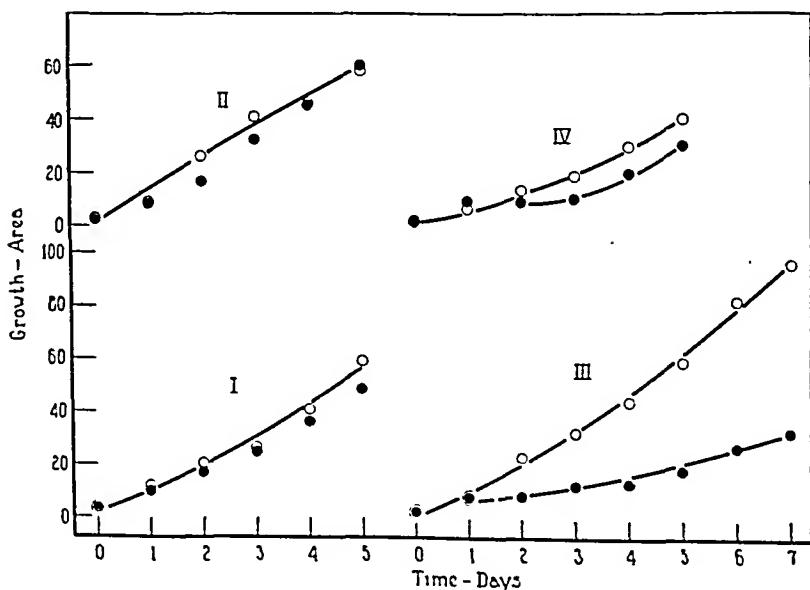


CHART 5. Growth of cultures exposed for varying intervals to 52°C.: I. Exposure time = $\frac{1}{2}$ minute; II. Exposure time = 1 minute; III. Exposure time = 2 minutes; IV. Exposure time = $2\frac{1}{2}$ minutes.

denominated an exposure as lethal when the culture exposed shows no growth after a 24 hour interval. We have verified the fact that cultures that cease to grow are really dead, by making transplantations of such cultures into fresh media. In no case was renewed growth observed, whereas all cultures showing continued growth after sublethal exposures did grow (and at a normal rate) on transplantation to fresh media.

The lethal exposure at 50°C. is between 5 and 6 minutes, whereas

growth inhibition is established definitely after 2 to 3 minutes of exposure. Again we notice that the manifestation of growth inhibition and death is not had until after 24 hours of growth (Chart 4). Our findings for exposures to 52°C. are similar, except that the lethal exposure is reduced to $3\frac{1}{2}$ minutes, the definitely inhibitory exposure to $\frac{3}{4}$ minute.

The findings for the five temperatures studied are summarized in Table I.

The data expressing the relation to temperature of the exposure time just necessary to induce growth inhibition can be fitted by the Arrhenius equation, and the value of the constant μ in this equation becomes $149,300 \pm$ calories. Such a high value is characteristic of processes involving thermal destruction (*cf.* Brown and Crozier, 1927;

TABLE I

Temp.	Growth inhibition after exposure		Death after exposure
	Observed	Calculated ($\mu = 149,300$)	
°C.	<i>min.</i>	<i>min.</i>	<i>min.</i>
42	?	—	?
44	180	215	?
47	30	24	105
50	2.5	2.7	6
52	0.75	0.65	3.5

Westermarck, 1927). The temperature coefficient for the actual death of the cultures in these experiments is also very high, but the data do not permit its accurate calculation. Most experiments on the "thermal death point" of tissues grown in culture are too scant to permit any reliable calculation of the temperature characteristic, though all indications are that it is very high. The chief concern of most experimenters has been to determine the exact lethal exposure time at temperatures just above normal. Friedgood (1928) for example, established that Walker rat sarcoma cells were killed by a 30 minute exposure to 44°C., but found no differences between the susceptibilities of the malignant cells of the tumor and the normal mononuclear elements. Other workers have also been concerned with the relative susceptibilities of malignant and normal tissues (for references see Friedgood, 1928; and Westermarck, 1927). We have been unable to discover, however, any detailed analysis of the growth of tissue

cultures in relation to temperature, particularly with reference to the inhibitory effect of sublethal exposures.

The only ascertainable morphological difference between cultures receiving sublethal and lethal exposures consists in a definite rounding up of the cells in the latter case. This is illustrated in Fig. 1 and is indicative of the death of the cells (*cf.* Kokott, 1930, *a*). Note the apparently flourishing condition of the culture receiving an exposure sufficient to induce marked growth inhibition (Fig. 2).

Lethal exposures, then, result in the death of the cells of the culture. But what factors govern the growth inhibition in sublethal exposures? We have ascertained that it is not a heat destruction of the growth factor in the embryonic tissue juice. Our experiment consisted simply of heating three samples of embryonic juice at 50°C. for 4 minutes, 5 minutes, and 7 minutes respectively. Tissue grown in media containing these samples as constituents showed no detectable alteration in its growth after 72 hours of growth, although an exposure of 7 minutes at 50°C. is sufficient to kill a culture and the shorter exposures induce marked growth inhibition.

It is possible that exposures to high temperatures caused the production of toxic substances in the blood plasma.¹ We have not tested this point, but are inclined to doubt the possibility. The experiments of Kokott (1930, *a*) indicate that exposures to supranormal temperatures result in a definite rounding up of the cells of a tissue culture. This rounding up is reversible with sublethal exposures but not with lethal exposures (see also Loeb and Drake, 1924). Exposures to high temperatures affect also the mitoses, reducing both the number of mitoses (Kokott, 1930, *b*) and the rate of mitotic division (Bucciantie, 1927). There is then a direct effect upon the cells of the culture. The high temperature coefficient that we obtain for the growth inhibition indicates a "destructive" process. Whether this destructive process involves the death of increasing numbers of cells with increasing temperature, or the destruction of increasing amounts of a substance necessary for normal cell growth, we cannot decide. It is interesting in this connection to note that Mossa (1927), studying the growth of neurites at various temperatures, was able to discover only a few

¹ It has long been known, of course, that heating embryonic tissue juice for 1 hour at 56°C. destroys its growth-promoting power. Similar heating of the blood plasma at 56°C. makes it strongly growth-inhibiting.

slowly growing fibers at 46°C. At 44°C., however, he did apparently observe the usual number of growing elements, but these showed a reduced rate of growth compared with the maximum at 39°C. A reduction in the rate of biological processes beyond certain definite critical temperatures has, of course, been repeatedly observed (*cf.* Crozier, 1926; Crozier and Stier, 1927).

SUMMARY

In a study of the growth of chicken osteoblasts in cultures exposed to supranormal temperatures of 42°C., 44°C., 47°C., 50°C., and 52°C., it has been observed that:

1. There is no lethal effect after prolonged exposures at 42°C. and 44°C.
2. Cultures are killed after an exposure of 105 minutes at 47°C., of 6 minutes at 50°C., and of 3.5 minutes at 52°C.
3. A definite inhibition of growth occurs after different exposures at all temperatures from 44°C. onwards (see Table I).
4. There is a latent period of approximately 24 hours before any discernible effect of sublethal or just lethal exposures; this latent period appears to be independent of the duration of the sublethal exposure and of the temperature.
5. The high temperature coefficients for lethal exposures and for exposures just sufficient to inhibit growth indicate an underlying "destructive" process in the cells of the culture.

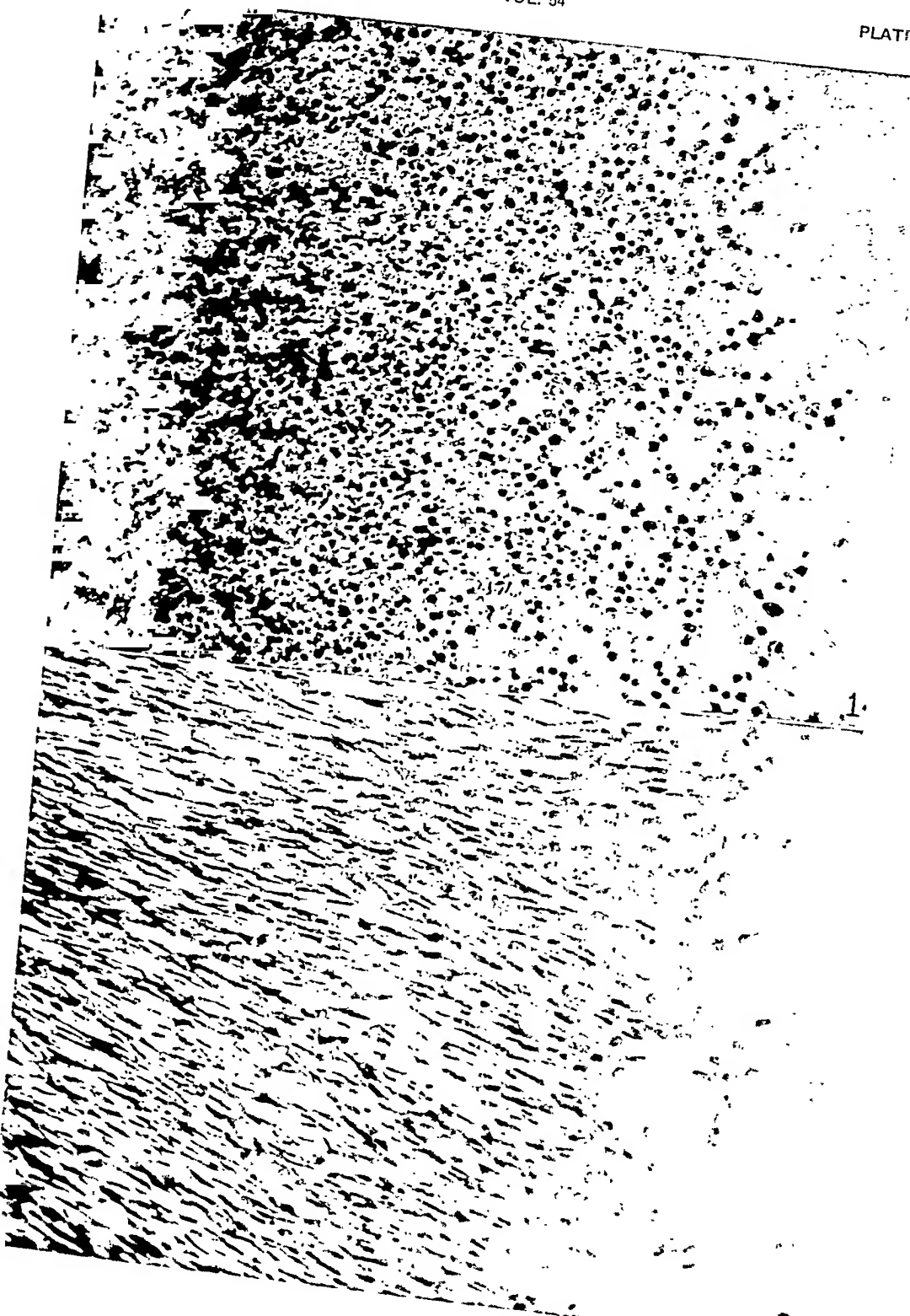
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EXPLANATION OF PLATE 29

FIG. 1. Microphotograph of a culture exposed to 50°C. for 7 minutes. The staining was done with Haidenhain's iron-hematoxylin. $\times 165$. See text.

FIG. 2. Microphotograph of a culture exposed to 50°C. for 4 minutes. The staining was done with Haidenhain's iron-hematoxylin. $\times 165$. See text.



1.

2

FIGURE 1. Tissue section from the brain of a patient with schizophrenia.

THE INDUCED DEVELOPMENT AND HISTOGENESIS OF PLASMA CELLS

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PLATES 30 AND 31

(Received for publication, June 4, 1931)

It is known that plasma cells occur normally in the connective tissues of warm blooded animals and that they are increased in numbers around various pathological lesions. In a study of the cellular reactions to the different chemical fractions of the tubercle bacillus, Sabin, Doan, and Forkner (1) reported the presence of these cells in rather large numbers in the omenta of rabbits after intraperitoneal injections of tuberculo-protein.

The present work was undertaken to determine the effectiveness of the tuberculo-protein as a stimulant for plasma cells; and to use the resulting proliferations of plasma cells for a study of their histogenesis.

If the plasma cell is accepted as a definite cellular entity, it must have a maturation cycle, as have the myeloid elements and the lymphocyte (2). The cellular reaction following intraperitoneal injections of tuberculo-protein has made it possible to follow the evolution of plasma cells from primitive undifferentiated mesenchymal cells, through maturity, into Russell body cells and other degenerative forms.

The name plasma cell was introduced by Waldeyer (3) in 1875. From his work it is not clear that he described a specific type of cell. A definite cell strain was first recognized by Cajal (1890 (4)) in the condylomata of syphilis. He named them *cells cyanophils*. Unna (1891 (5)) found cells in the lesions of dermal tuberculosis which he called plasma cells. He described them as having a basophilic, spongy, or granular cytoplasm, the granuloplasm. Their nuclei were either centrally or eccentrically placed, and the chromatin content and arrangement were variable. His only constant specializing criterion was basophilic granuloplasm, and as a result of this he included under the category of plasma cells many cells with basophilic cytoplasm, which were probably derivatives of other strains.

Marshalkó (1895 (6)) selected certain morphological characteristics as the essential criteria of the typical plasma cell. By his definition such a cell must have basophilic cytoplasm, showing a clear area in the center, be round or oval, have a round, eccentrically placed nucleus with the chromatin condensed near the nuclear membrane and arranged radially in blocks. Cells that have these criteria are known today as typical plasma cells. It was Marshalkó's belief that they originated from hematogenous lymphocytes.

Downey's (7) discussion of the controversy over the origin of plasma cells shows that many believe them to be derived from lymphocytes, either hematogenous or histogenous, while others think they take origin from primitive mesenchymal cells. He gives evidence to support the view that they are derived from both types of lymphocytes, but in greater numbers from the hematogenous variety. In describing plasma cells in the mesentery of the frog, he shows that the Russell body cells are the end stage of their development and are present normally. The Russell body cells are plasma cells which have fulfilled a cellular secretory function, having built up some material in their cytoplasm, which first appears as granuloplasm, and later, becoming hyalinized, is present as the Russell bodies. An excellent review of the literature has been given recently by Michels (8).

Although plasma cells are found in normal connective tissues, they are present in the greatest number in organs which act as bacterial filters or which presumably have a detoxifying function, notably the lymph nodes and omentum. This, together with their presence in certain pathological lesions, suggests that they act to detoxify bacterial and other toxins.

Methods

Three tuberculo-proteins were used to stimulate the production of plasma cells, as follows: (a) one isolated by Johnson and Coghill (9), designated water-soluble 30±; (b) an alkali-soluble fraction, also isolated by Coghill (10); and (c) a water-soluble material similar to that of Johnson but prepared by the Mulford Company (MA-100). The first two, because they were slightly acid, were suspended in distilled water or salt solution and caused to dissolve by the addition of enough 1 per cent NaOH to give a neutral reaction to litmus. The Mulford protein, MA-100, was received in salt solution and injected in this form.

Eighteen animals were employed in the experiments. To stimulate the cells in the omentum, seventeen of them were given intraperitoneal injections of one of the proteins. Two of the seventeen animals were also given intravenous injections, and three were injected subcutaneously. The remaining animal was given intravenous injections only.

Table I indicates the number of injections, the type and amount of protein each animal received, and the interval between the last injection and autopsy.

After its course of injections, each animal was killed by intravenous injection

of air and an autopsy was performed. The omentum was spread over a slide prepared with neutral red and Janus green for study by the supravital method. Lymph nodes were scraped and prepared for supravital study. Parts of the various organs which seemed to be injured by the action of the protein were fixed in Helly's solution and then they were stained with hematoxylin and eosin, or with Giemsa, for microscopic study.

TABLE I

Rabbit No.	Type of protein	Number of injections	Amount of each injection	Interval between last injection and autopsy
			mg.	days
R 1536	304	3	20	7
R 1166	304	3	20	10
R 1734	Alkali-soluble	5	10	7
R 1803	Ma-100	5	20	11
R 1600	304	4	20, ip. 10, iv.	2
R 1105	304	6	20	2
R 1735	Alkali-soluble	10	10	6
R 1151	304	12	10	2
R 1153	Alkali-soluble	12	10	2
R 1112	304	7	14, ip. 6, sc.	5
R 1109	304	10	20	1
R 1104	304	12	20	3
R 1804	MA-100	13	20	21
R 1106	304	15	18, ip. 6, sc.	4
R 1102	304	20	20	7
R 1168	Alkali-soluble	25	10, ip. 5, sc. (5 injections)	2
R 1608	304	36	10, iv.	2
		10	10, ip.	2
R 1211	304	48	10, iv.	Not dead

Induced Foci of Plasma Cells

At autopsy the omentum usually showed a hyperplastic reaction; it was thickened, had an increase of milk spots, as well as much new fibrous tissue and many new blood vessels. In several of the animals (R 1102, R 1168, R 1153, R 1735¹) small white nodules were found

¹ These are serial numbers of the work of the department covering a term of years.

attached to the omentum, body wall, and large intestine. Often there was inflammation of the body wall, with a new growth of small vessels and a roughening of the whole surface.

The omenta of five normal rabbits (R 650, R 651, R 652, R 578, and R 879) were studied in comparison with those of the experimental animals. Plasma cells were found in only two of these and in rather small numbers. The greater part of each normal omentum, as seen in section, was made up of fat cells. The remainder of the cellular structure was made up of undifferentiated cells, small groups of lymphocytes, a few monocytes, and a network of mesothelial cells.

Table II indicates the organs in which plasma cells were found in the experimental animals. The omentum was the site of the greatest proliferation of these cells, although they were present in the liver, spleen, cecum, body wall, and scattered lymph nodes. Two animals which received intravenous injections also showed a few plasma cells in the bone marrow. In the omentum the plasma cells were found in the milk spots, about the blood vessels, and scattered through the connective tissue. Some omenta were so stimulated by the protein that the plasma cell was the predominating cell element (Fig. 5). Where they were so vastly increased in numbers, the lymphocytes were also increased. New lymph follicles with germinal centers could be made out where the irritation had been greatest. Plasma cells were not seen in these lymph follicles, although they often encroached on the borders of them. On the whole the two strains of cells were quite definitely segregated, but one animal (R 1168) had areas in the omentum in which plasma cells and lymphocytes were mixed. The lymphocytes, as a rule, lay in the spaces between the fat cells, and the plasma cells always in the diffuse connective tissue.

The white nodules noted on the omentum, body wall, and large intestines were made up of necrotic material. Many of the cellular elements were still recognizable in them as plasma cells and leucocytes. The tissue surrounding these nodules was usually made up of connective tissue cells, including many plasma cells. One such nodule was taken from the large intestine of R 1735; another from the large intestine of R 1153. In these could be seen three stages of the growth of new fibrous tissue. There were areas of degenerating plasma cells and leucocytes, areas of deposition of fibrin and slight hemorrhage,

TABLE II
Organs in Which Plasma Cells Were Found

Rabbit No.	Omentum	Kidney	Liver	Spleen	Body wall	Small intestines Peyer's patches	Wall of large intes- tines	Bone marrow	Lung	Lymph nodes						Cecum
										Axillary	Submaxillary	Popliteal	Inguinal	Retroperitoneal	Tracheal	Mesenteric
R 1112	+	0	+	0	+	0	+	0	0	0	+	0	0	0	0	0
R 1153	+	0	+	0	+	0	+	0	0	0	+	0	0	0	+	0
R 1105	+	0	+	0	+	0	0	0	+	0	+	0	0	0	+	0
R 1151	+	0	0	+	+	0	0	0	+	0	+	0	0	0	+	0
R 1106	+	0	0	0	+	0	0	0	+	0	+	0	0	0	+	0
R 1101	+	0	0	0	+	0	0	0	+	0	+	0	0	0	+	0
R 1109	+	0	0	0	+	0	0	0	+	0	+	0	0	0	+	0
R 1536	+	0	0	0	+	0	0	0	+	0	+	0	0	0	+	0
R 1102	+	0	0	0	+	0	0	0	+	0	+	0	0	0	+	0
R 1680	+	0	0	0	+	0	0	0	+	0	+	0	0	0	+	0
R 1731	+	0	0	0	+	0	0	0	+	0	+	0	0	0	+	0
R 1168	+	0	0	0	+	0	0	0	+	0	+	0	0	0	+	0
R 1735	+	0	0	0	+	+	+	0	0	0	+	+	0	0	+	0
R 1166	+	0	0	0	+	+	+	0	0	0	+	+	0	0	+	0
R 1608	+	0	0	0	+	+	+	0	0	0	+	+	0	0	+	0
R 1503	+	0	0	0	+	+	+	0	0	0	+	+	0	0	+	0
R 1504	+	0	0	0	+	+	+	0	0	0	+	+	0	0	+	0

and areas of new fibrous tissue. About the base of the gland cells were many lymphocytes and many young plasma cells. One nodule from the body wall of R 1168 showed an infiltration of the plasma cells into the connective tissue between the muscle bundles. The lung of R 1536 contained an area of bronchopneumonia about which was a small amount of fibrous tissue. In this fibrous tissue and infiltrating into the pneumonic area were many plasma cells. There were also present small nodules of lymphocytes with germinal centers.

The animals which received the largest amounts of the protein over the longest periods of time (R 1102, R 1109, and R 1168) showed the greatest proliferation of plasma cells. These animals also revealed the greatest variety of stages in the life cycle of the plasma cell. The period elapsing between the last injection and the autopsy was a determining factor in the differentiation of the plasma cell. More true Russell body cells were found in the tissues of those animals, especially R 1804, in which this period was the longest. The largest numbers of degenerating plasma cells were not necessarily present in these animals, but time was probably not so much responsible for them as was crowding.

Histogenesis of Plasma Cells

Most of the plasma cells in both the stimulated tissues and the normal organs were of the typical Marshalkó variety. However, other plasma cells with definite characteristics of immaturity were always seen in or near all the groups of typical forms. There were also older degenerating cells, but these were rare except in the necrotic white nodules which were composed of them almost wholly.

The primitive mesenchymal cells and the young cells in the milk spots were the precursors of the plasma cells. Undifferentiated mesenchyme cells are normally present scattered diffusely in the connective tissues and lying along the blood vessels. The milk spots of the omentum are made up chiefly of relatively undifferentiated cells which have the potentiality of developing into monocytes or macrophages. These cells have been termed polyblasts by Maximow (11). Sabin *et al.* (1) have shown them to be transformed readily into monocytes after intraperitoneal injections of the tuberculo-phosphatide. Monocytes, lymphocytes, and cells which are more immature

than these so called polyblasts are also found in the milk spots. These young cells in the milk spots also are often called primitive cells. In stained sections they appear slightly younger than blast forms of lymphocytes and leucocytes.

Two primitive mesenchymal cells are shown in Fig. 1 (see arrows). These cells were characterized by large nuclei and little if any cytoplasm. The structure of the nuclei was a fine network with little chromatin. This stained light gray-blue and the nucleoli, which were small and usually two or three in number, stained red or purple. When the cytoplasm was discernible, it was of a slightly basophilic quality. The development of the primitive cells into the Marshalkó type plasma cells was a graded transitional process in which both the nuclei and cytoplasm changed in staining quality and structure. The fact that the young cells were of different ages could be readily made out. Those slightly older than the primitive cells showed slight condensations of chromatin near the nuclear membranes. The nucleoli of these cells were somewhat larger than those in the undifferentiated cells, and the cytoplasm showed beginning basophilia. The grade of basophilia of the cytoplasm and the amount of condensation of chromatin of the nuclei were indices of how far advanced from the primitive cell any one of these had become. Many of this type of cell are shown in Fig. 1. The photograph was taken from a section of body wall of R 1168. A portion of the section was composed almost entirely of young cells and there were also a few lymphocytes. The part shown was from a nodule in the body wall which was almost tumor-like in its cellular picture. Typical plasma cells were seen in groups and scattered throughout all parts of the tissue surrounding the nodule.

As the young forms matured, they fulfilled the criteria of the Marshalkó plasma cells. Young cells were seen which had one or all of the specified characters. Some had a distinctly basophilic cytoplasm with a clear area in its center. The chromatin in the nucleus was so condensed as to give the appearance of the cart-wheel nucleus, a *Radkern*, and in most of this type of cell the nucleoli were retained. The nuclei were either eccentric or central in position.

In groups of the young plasma cells some were found with mitotic figures; one such is shown in Fig. 1. Reproduction of the plasma cell in this early phase by mitosis, though not rare, was not very common.

As the cells matured, the nuclei became smaller and the chromatin more condensed, especially at the nuclear membrane. The condensation of the chromatin was in blocks which gave the characteristic cart-wheel nucleus. The basophilic quality of the cytoplasm became more pronounced. Most of this type of cell had eccentrically placed nuclei and clear areas in the centers of their cytoplasm. In these mature cells the nucleoli were lost. These cells are shown in Fig. 2. In this figure are seen typical plasma cells and also two or three cells which have one or more of the criteria of Marshalkó plasma cells, but which are too young to have them all. There are also areas such as that represented in Fig. 5, in which all the cells are of the mature type. The plasma cells proliferated by both mitosis and amitosis, and this latter fact is shown by the presence of two or three nuclei, or by large cells budding smaller ones.

Even in the mature plasma cells, gradations of morphological character were evident. Not only were some obviously younger than the typical Marshalkó variety, but there were also older ones. The cytoplasm of these latter had become spongy and somewhat granular. The spongy cytoplasm conformed to the description of granuloplasm by Unna. The nuclei of cells with granuloplasm were usually smaller than those of the less mature cells. The cytoplasm of the younger cells, whether of the pre-Marshalkó type or of the Marshalkó type, appeared homogeneous in contradistinction to the granuloplasm.

Following the development of granuloplasm, the plasma cells began to degenerate. Two types of degeneration occurred. Some cells, as they became senile, lost their basophilic appearance, the nuclei became pycnotic, and often vacuoles appeared in the cytoplasm. There developed in certain of these cells hyaline bodies with the same appearance and staining reactions as the true Russell body cells. Most of the cells undergoing degeneration of this first sort were packed closely together, and it is possible that crowding led to their degeneration. The other type of degeneration was into characteristic Russell body cells. Some of the plasma cells developed acidophilic granules and hyaline or crystalline bodies appeared later in the cytoplasm; each of these bodies seemed to be a segregated part of the latter. The nuclei were fragmented or pycnotic and usually were in the eccentric position. The true Russell body cells were found infrequently in any of the areas

which had been stimulated with the protein. The first type of degeneration was frequent wherever the plasma cells predominated in the tissues, as in the white nodules described above. True Russell body cells could be made out scattered through the matrix of aggregates of the sort. Fig. 3 shows degenerating plasma cells. A few of these contain acidophilic granules and hyaline bodies (Arrows A).

Supravital Studies

Supravital studies of plasma cells have been reported by Bloom (12), Forkner (13), Jackson *et al.* (14). Their descriptions are quite in agreement, except for the fact that Jackson did not find neutral red bodies in the cytoplasm constantly.

Omenta from normal rabbits and from rabbits injected with tuberculo-protein were examined with the supravital method. The stimulated omenta were studied first and the findings correlated with those of the fixed tissues. All omental spreads were fixed with methyl alcohol and stained with methylene blue and Giemsa after they had been studied by the supravital technique.

The plasma cells appeared round or oval, when stained supravitaly, or they took on peculiar configurations when pressed by other cells. The cytoplasm was a yellowish gray and, in the spreads, the mitochondria were not seen unless a small amount of a 1:10,000 solution of Janus green was added. Mitochondria did not always stand out even after this treatment. When demonstrable, they were either scattered throughout the cytoplasm, or were grouped in the center of it. Neutral red bodies were rare. Occasionally, in very large cells, a thinning in the center of the cytoplasm represented the clear area so characteristic in fixed staining. The mitochondria were usually numerous, some cells having as many as 30 or 40 of them. They varied in size and in shape and were usually smaller than those in young lymphocytes. They took the forms of small rods or round dots, though the rod-shaped mitochondria were more frequently seen.

The nuclei of these cells were usually about the same shade of gray as the cytoplasm. This made them difficult to see, though the heaping up of the chromatin was often distinctive. If the cells were young enough, nucleoli stood out as small pearls. Differentiation of young cells from older ones was difficult to make out, because the charac-

teristics of age were not marked in preparations made by this technique, except in the case of the Russell body cell.

Russell body cells were found in the tissues of three of the experimental animals. The cytoplasm of these cells appeared as gray as the cytoplasm of the younger cells. Some of the Russell body cells had from three to five cytoplasmic lobulations; others had lobulations which entirely filled the cells. Each lobulation seemed to be a segregated part of the protoplasm, and was smooth and non-granular. The nuclei were always eccentric in position and usually smaller than those of the less mature cells. The cells with the larger number of lobulations had no mitochondria, and the nuclei were so small that they could not be seen or else possibly were directed away from the microscope.

The cells described here as plasma cells were found singly and in groups throughout all stimulated omenta and were usually beneath the surface, so that they were difficult to bring into focus. They were most numerous along the blood vessels and in the milk spots. Lymphocytes were also seen, but as already mentioned, they were rarely in the groups of plasma cells.

The two animals which received intravenous injections of the protein developed as many as 4 per cent plasma cells in the peripheral blood stream on several occasions. Here they had the same tinctorial properties as in the omental spreads, but they usually contained from five to eight neutral red bodies grouped in the center of the cytoplasm. Maximow (11) has previously shown that the clear area is the site of the centrosome, and it is not therefore peculiar that there was a grouping of the vacuoles stained with neutral red around this space. The mitochondria stained more brightly and were seen with less difficulty than in the omental spreads. They were usually scattered throughout the cytoplasm. The nucleus often was very indistinct, being of about the same shade of gray as the cytoplasm.

Unstimulated omenta also contained cells which were recognized as plasma cells. In these omenta they were found rarely, although one animal (R 1606) had many of them.

By the supravital technique, plasma cells were differentiated from lymphocytes by their darker gray cytoplasm, eccentric nuclei, and the variations in size, staining quality, and distribution of mitochondria.

In the young lymphocytes the nuclei were usually large and the mitochondria stained with ease and were grouped about the periphery of the nucleus. The nucleus of the young lymphocyte stood out plainly, while that of the plasma cell was usually indistinct.

The plasma cells in these same omental spreads, when stained with methylene blue and Giemsa, were for the most part of the Marshalkó type. The cytoplasm of many of them had a tendency to be granular. Russell body cells had the same appearance as in tissues stained with hematoxylin and eosin. The nuclei of the typical plasma cell showed an unaltered chromatin arrangement, but stained light pink. As by the supravital technique, these cells were grouped about blood vessels, in the milk spots and scattered throughout the connective tissue stroma. In some parts of each omentum many lymphocytes were found, but only rarely amongst the plasma cells.

Some omenta showed increases in the number of fibroblasts and clasmatocytes. In these, often, the fibroblasts were seen with their fibrils about single plasma cells. This peculiar relationship is shown in Fig. 4.

Lack of Relationship of Plasma Cells and Lymphocytes

In many aspects plasma cells and lymphocytes resemble each other. Plasma cells are found where lymphocytes develop, and the stimulating effect of the tuberculo-protein causes many young lymphocytes and new lymph follicles, as well as plasma cells, to be formed. The plasma cells are found almost always in the connective tissues; even when found in the lymph nodes they are in the connective tissue cords. They are a constant element in the submaxillary lymph nodes and occasionally may be present in any other of the lymphoid structures (Table II). In the submaxillary nodes they are usually present in rather large numbers, while in other nodes they are few in number.

As a rule, plasma cells are absent from the popliteal and inguinal nodes. Because of this, and the fact that lymphocytes have been termed the precursors of plasma cells, these nodes were subjected to direct stimulation or irritation by the protein. To effect this stimulation of the inguinal nodes, two animals were injected subcutaneously in the groin. Another animal was given injections of the protein into its left foot pad so that the material would drain through the popliteal node.

R 1106 received fifteen injections of the material in the right groin, each of 6 mg. in 2 cc. of fluid. R 1112 received five similar injections in the right groin. R 1168 was given five injections of 5 mg. each in its left foot pad. When these animals were autopsied, supravital studies of the regional lymph nodes showed no plasma cells. The inguinal nodes of R 1106 were very much enlarged and contained epithelioid cells, but those of the other animals were not enlarged and were free from abnormal components.

The fixed sections of the right inguinal nodes of R 1106 contained few plasma cells, and there were a few scattered plasma cells in the left popliteal nodes of R 1168. The other regional nodes or those which were subjected to the irritation of the protein were free from plasma cells.

Young plasma cells differ much from young lymphocytes in their morphology. The nuclei of young plasma cells are denser in chromatin; they simulate the nuclei of the older cells, but are larger and usually contain nucleoli. The primitive cells, of course, are so undifferentiated that they have no specializing criteria. Primitive cells which form plasma cells are much like those that form lymphocytes. The cytoplasm of the youngest differentiated cells of the plasma cell series is usually basophilic, although not so markedly as that of older cells. Young lymphocytes appear much like the young plasma cells, although their nuclei are centrally placed, and often bean-shaped. The chromatin in the nuclei of the young cells of the lymphocyte series is usually in thread-like formations, not condensed near the nuclear membranes. The cytoplasm of these cells is slightly less basophilic than that of the plasma cells. Mitotic figures are more frequently seen in groups of young lymphocytes than in plasma cells.

Young lymphocytes are best seen in or near the germinal centers of the lymph nodes. The mesenteric nodes contain many more young forms than do other nodes, and they are abundant in the sinuses. Where these young cells are found there are no plasma cells of the Marshalkó type.

There is no difficulty in discriminating Marshalkó plasma cells and the degenerating forms from lymphocytes of all types. It has been a rule to reserve the name plasma cell for these mature forms. However, many workers have included abnormal types of cells in this category. This should not be done unless these cells have one or several of the characters of true plasma cells, and show by proximity that they are developing into more mature typical forms. The differential

characters of the young cells of the lymphocytic and plasma cell series are definite though not marked. This, together with the fact that few plasma cells developed in organs rich in lymphocytes after direct stimulation of those organs, makes it clear that plasma cells are not derived from lymphocytes. With a separation of the young cells into two classes the maturation cycle of the plasma cell is complete.

SUMMARY AND CONCLUSIONS

As result of finding numerous plasma cells in the omenta of rabbits injected with tuberculo-protein, a method to induce the production of large numbers of these cells has been discovered. The tissues in which they were pronouncedly increased were the subserosal connective tissues of the omentum, body wall, and intestinal wall.

The precursor of the plasma cells is a primitive connective tissue cell. As this cell develops into the typical Marshalkó plasma cell there is a progressive increase in the basophilia of the cytoplasm, the nucleus becomes eccentric, a condensation of the chromatin occurs near the nuclear membrane, and there is a loss of the nucleoli. At the time when the nucleus assumes the eccentric position, the clear area appears in the center of the cytoplasm. The early cells are capable of reproducing themselves by mitosis, while the typical mature cells divide by amitosis.

The mature plasma cells often have muddy, spongy cytoplasm which contains acidophilic or hyaline granules as the cells grow old or begin to degenerate. The cells with granules or hyaline bodies usually have pycnotic or fragmented nuclei. These cells are the final stage reached by some plasma cells. Others, when degenerating, show vacuoles and signs of senility. Those with the granules and hyaline bodies are the so called Russell body cells.

Plasma cells developed in greatest numbers after our largest injections of tuberculo-protein. The differentiation into young, mature, and senile forms was most clearly recognizable when some days had been allowed to elapse after the last large injection of the stimulating agent.

A description of the plasma cell as viewed supravitally has been given. The cells are met in the blood stream as well as in the tissues. They are characterized by their deep yellowish gray cytoplasm, in-

distinct eccentrically placed nuclei, and large numbers of mitochondria.

The plasma cells differ from lymphocytes, in that they did not develop in large numbers after direct stimulation of the lymph nodes with tuberculo-protein. The young plasma cells also differ in morphology from the young lymphocytes. When plasma cells were found in the lymph nodes they were in the connective tissue cords.

The plasma cell is a definite entity, having a maturation cycle. It is stimulated to great proliferation by certain toxic irritants.

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EXPLANATION OF PLATES

PLATE 30

FIG. 1. Section from body wall of Rabbit R 1168 which received twenty-five intraperitoneal injections of 10 mg. each of alkali-soluble protein. It shows many young plasma cells; two are primitive connective tissue cells (Arrows), many have eccentrically placed nuclei, and the chromatin of most of the nuclei shows a beginning condensation near the nuclear membrane. Hematoxylin and eosin. $\times 1,000$.

FIG. 2. Section from omentum of Rabbit R 1102 which received twenty intraperitoneal injections of 20 mg. each of 304 protein. It shows plasma cells of the typical Marshalkó variety. The arrow points to a cell assuming the characters of the mature form. Hematoxylin and eosin. $\times 1,000$.

FIG. 3. Section from the wall of the large intestine of Rabbit R 1153 which received twelve intraperitoneal injections of 10 mg. each of alkali-soluble protein.

It shows degenerating plasma cells, including some cells which contain Russell bodies. Arrows labeled A point to almost typical Russell body cells, while Arrows B indicate cells with acidophilic granules. These cells are less mature than the typical Russell body type. Hematoxylin and eosin. $\times 1,000$.

FIG. 4. Omental spread taken from Rabbit R 1166 which received three intraperitoneal injections of 20 mg. each of 304 protein. It shows four plasma cells, one of which has about it the fibrils of a fibroblast. It was first studied in supravital neutral red and Janus green, then fixed in methyl alcohol and stained with methylene blue and Giemsa. $\times 1,000$.

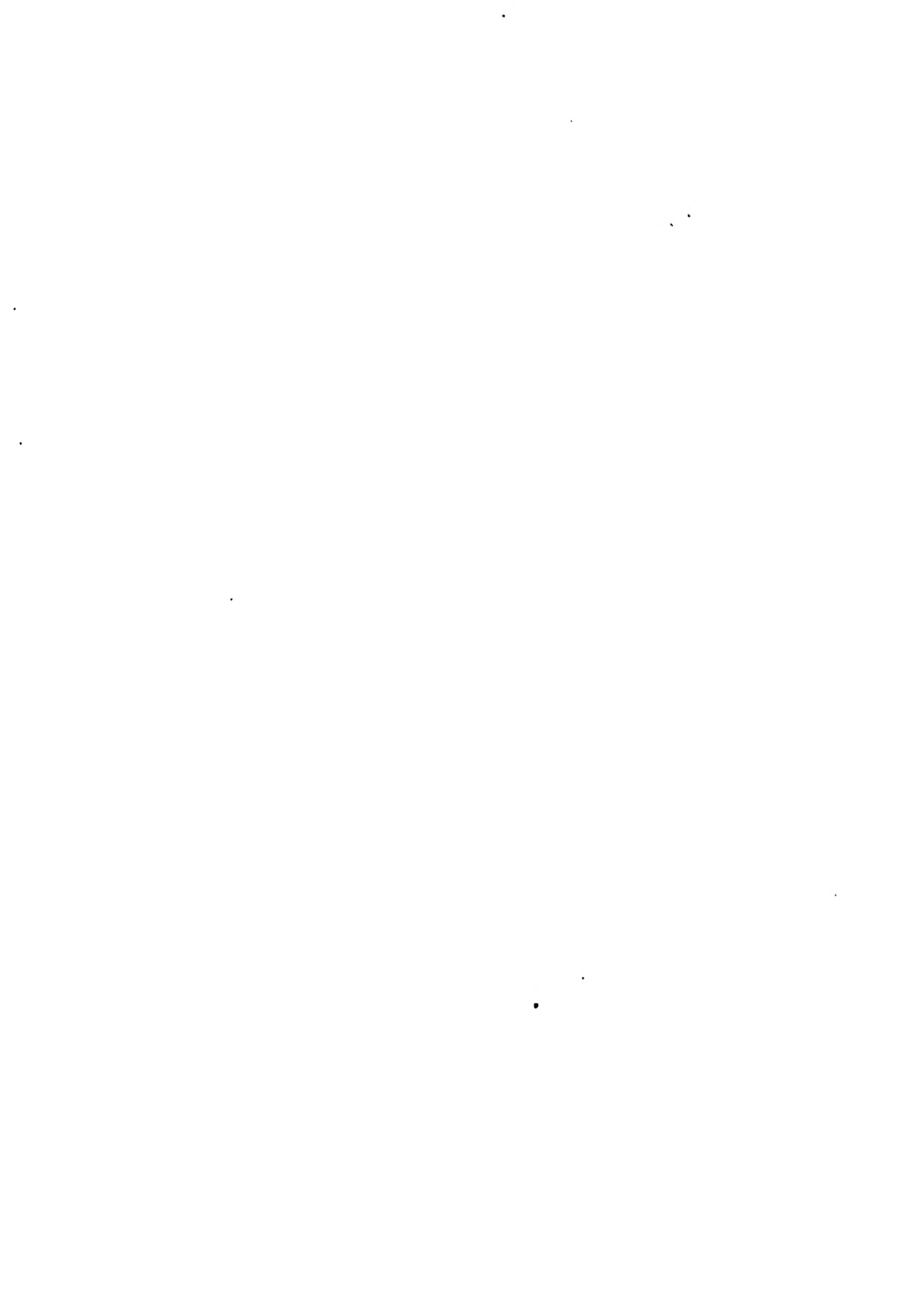
PLATE 31

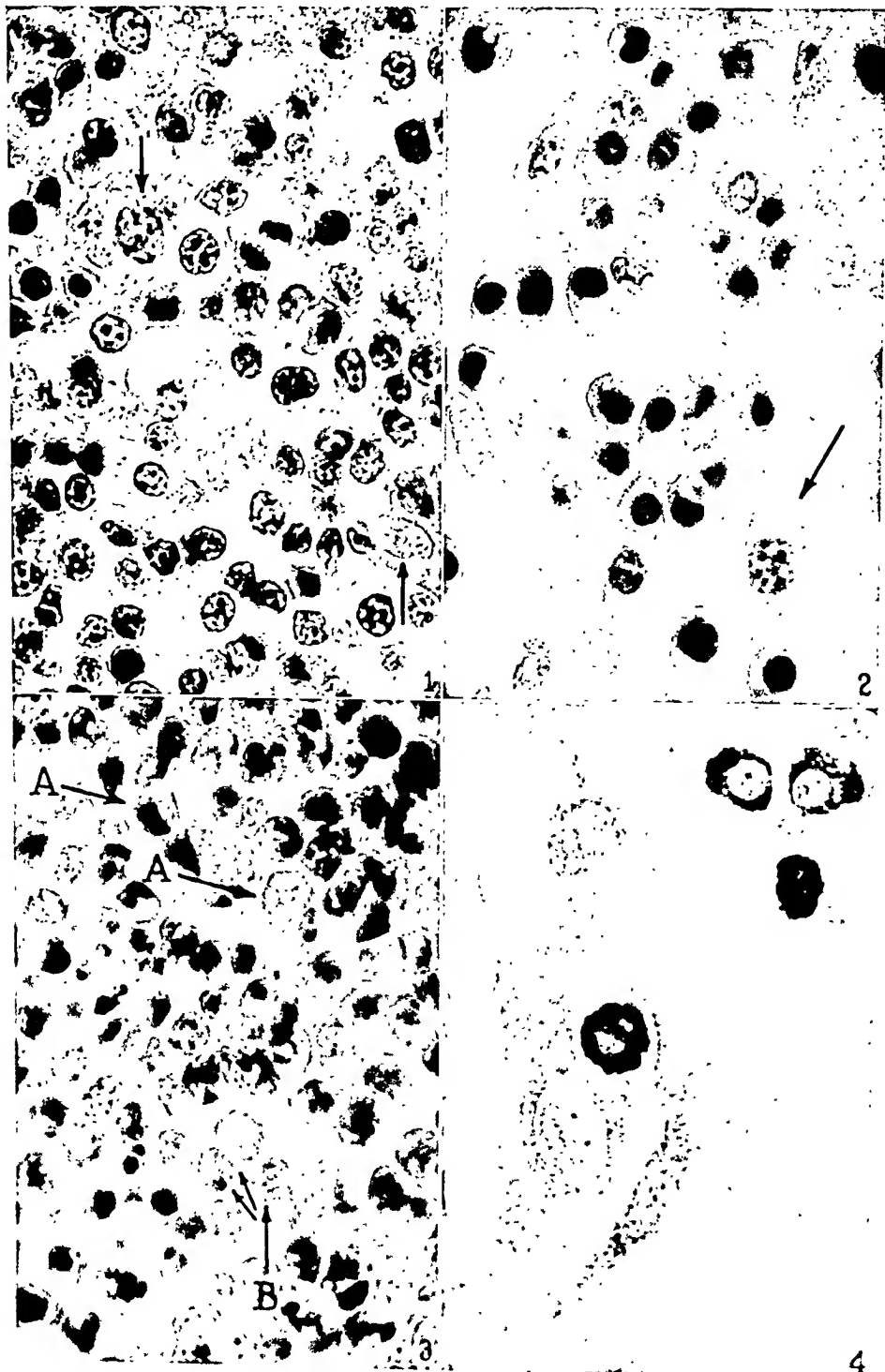
FIG. 5. Section of omentum of Rabbit R 1102 which received twenty intraperitoneal injections of 20 mg. each of 304 protein. It shows a focus of typical plasma cells of the mature form. Hematoxylin and eosin. $\times 1,000$.

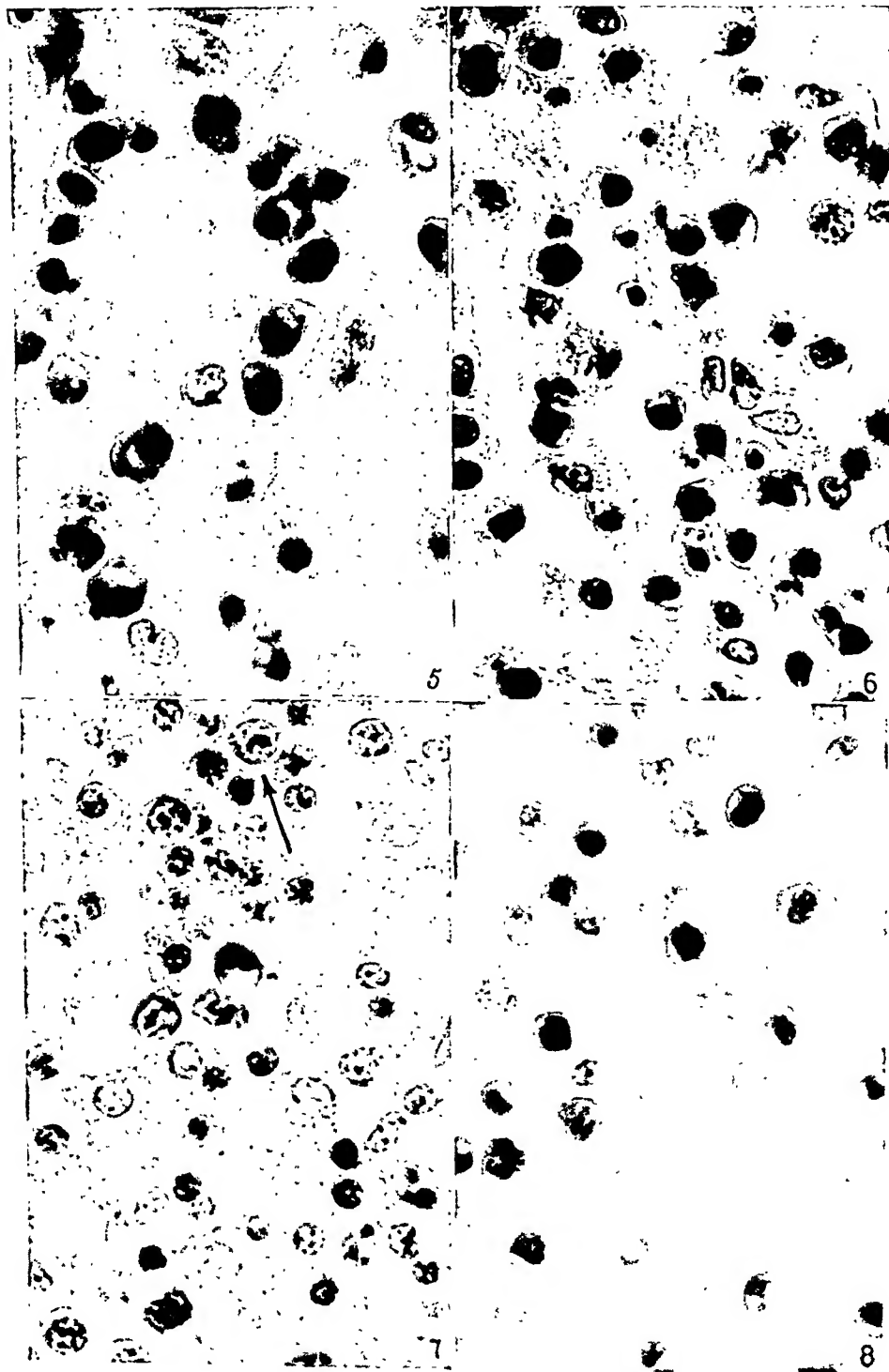
FIG. 6. Section of omentum of Rabbit R 1168 which received twenty-five intraperitoneal injections of 10 mg. each of alkali-soluble protein. This figure shows plasma cells in several of the phases of maturation. One cell in the lower right corner is budding a smaller cell, a mitotic figure is present above the center to the left, and there are several young cells scattered through the figure. Hematoxylin and eosin. $\times 1,000$.

FIG. 7. A portion of the same section shown in Fig. 1. It has many young plasma cells, one (Arrow) just beginning to differentiate from the primitive type. In the background are young connective tissue cells and clasmotocytes. Two large pale clasmotocytes are plainly seen near the upper right border. Hematoxylin and eosin. $\times 1,000$.

FIG. 8. Section of the same material as Fig. 5, but from a less cellular part. It shows scattered plasma cells, some more mature than others, and several long fibroblasts. Hematoxylin and eosin. $\times 1,000$.







SWINE INFLUENZA

I. EXPERIMENTAL TRANSMISSION AND PATHOLOGY

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PLATES 32 TO 34

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Swine influenza ("hog flu") was first recognized as a clinical entity in the fall of 1918. Because of the prevalence at the same time of human influenza and a marked resemblance in the symptoms of the two diseases Koen became convinced that they were the same. He therefore gave the name of "flu" to the new malady of hogs (1).

The cardinal features of swine influenza are amply presented in the veterinary literature (1-6).

Swine influenza is essentially a disease of autumn and early winter and reaches epizootic proportions each year. The onset is sudden and the incidence in an affected herd is practically 100 per cent. Fever, anorexia, prostration of an extreme type, cough, and a peculiar abdominal type of respiration are salient features of the disease. The animals cry out when handled, which has been interpreted as evidence of muscular tenderness. The period of illness is short, varying from 2 to 6 days, and in uncomplicated cases the recovery is almost as sudden as the onset. The mortality is stated to range from 1 to 4 per cent. Fatal cases exhibit an extremely edematous type of bronchopneumonia.

During the autumn of 1928 and of 1929 two epizootics were observed by the writer in eastern Iowa.¹ That in 1928 was very severe,

¹ For material and advice about the disease we are grateful to the following veterinarians of eastern Iowa: Drs. Fred J. Crow, J. S. Potter, and E. O. Thomas of Iowa City; A. H. Legenhausen, G. B. Munger, and J. W. Griffith of Cedar Rapids; H. J. Fry of Kalona, G. Lames of Dysart, J. B. Bryant of Mt. Vernon, R. Schuchert of Keystone, J. C. Glenn of Norway, and R. E. Elson of Vinton; also to Mr. N. W. Brooks of Cedar Rapids.

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and while the mortality as a whole probably did not exceed 4 per cent, in some herds the losses were more than 10 per cent. In 1929 the epizootic was extremely mild and the mortality was less than 1 per cent.

The term "hog flu," as popularly used, embraces more than one clinical entity. This point was particularly emphasized by our 1929 field observations. While in 1928 the epizootic disease was so plentiful in eastern Iowa that material from typical cases was easily obtained, in 1929 much difficulty was encountered. Many cases were then seen in which a loose diagnosis of "hog flu" had been made. These animals, as a rule, were found to be suffering from a respiratory affection, quite often simulating the true epizootic swine influenza in some respects but differing markedly in others. The true nature of the condition could usually be recognized by the absence of prostration on the part of the affected animals, the greater chronicity of the disease, and the failure of more than a small portion of the herd to become affected.

Experimental Transmission

Swine influenza has been established without difficulty by experimental infection of swine with eight separate strains of the disease brought from Iowa.

Infectious material was transported in tubes packed in iced thermos jugs and was usually *en route* for from 36 to 48 hours. The method used in inducing infections was that described by McBryde, Niles, and Moskey (6). It consisted in the intranasal instillation of either suspensions of bronchial mucus alone or mixtures of suspensions of bronchial mucus, bronchial lymph nodes, and diseased lung. The instillations were made with a Luer syringe without needle by pressing the tip of the syringe into the external nares. The suspensions were prepared in either distilled water or infusion broth and were made up to between 10 and 20 per cent. The dosage of suspension administered usually ranged from 5 to 30 cc., but typical and severe influenza was produced by the intranasal injection of doses as small as 1 cc. Only a small portion of the material administered was retained, for most of it was either sneezed or blown out of the nostrils.

After the establishment of the disease animals from which material for further inoculations was to be taken were slaughtered on the 3rd or 4th day following the first appearance of a temperature in excess of 40°C. The disease has been maintained for study by this type of serial passage.

Because of the highly contagious nature of the disease it has been necessary

to exercise extreme precaution in the isolation of individual experimental animals. Our individual isolation units have been used. We have followed the technique of isolation used here for the study of experimental hog cholera. This practice has been tested repeatedly for both hog cholera and swine influenza and found sufficient to insure complete isolation.

Unlike McBryde and his coworkers (6) we have encountered no spontaneously immune animals during the investigation. The probable explanation is that McBryde investigated the disease during the epizootic period in Iowa and used as his experimental animals swine from the same region. These animals may have been in contact with the disease prior to their use experimentally and may in consequence have developed some degree of immunity. Such a possibility was eliminated in our own experiments. All of the transmission work was conducted at Princeton, New Jersey, and the experimental animals were either raised on the Institute farm or purchased from nearby farmers. Epizootic swine influenza does not occur in the locality.

The method of inoculation has been adequately controlled. Normal swine have been injected intranasally with large doses of suspensions of lung and bronchial lymph nodes from normal swine and in no instance has any evidence of illness developed or anything suggestive of influenzal disease been encountered at autopsy.

Clinical Features of Experimental Swine Influenza

The symptoms induced by the several strains of swine influenza have varied in degree but very little in type.

Strain 1 (1928) induced a more severe and virulent swine influenza than Strain 2 (1928) and Strain 2 in turn was more virulent than Strains 5, 6, 7, and 10 (1929). Strains 14 and 15 (1930) induced a disease which was of about the same severity as that induced by Strain 1. The actual mortality of the experimental disease was not determined, for most of the experimental swine were killed on the 2nd to 4th day of fever for bacteriological and pathological studies. However, fatal infections resulted five times following infection with Strain 1, four times following infection with Strain 14, and six times following infection with Strain 15. The mortality can be safely estimated as higher than that in the naturally occurring field disease.

The incubation period was found to be short. For animals infected by pen contact it varied from 2 to 7 days with the average at 4 days. Animals infected by intranasal instillation became ill more promptly and usually within 24 to 48 hours exhibited a sharp rise in temperature.

Fever, *i.e.*, a temperature of 40°C. or higher, was in all cases the first observable evidence of illness. Accompanying the rise in temperature, or following it very shortly, there was a mild degree of malaise, mild anorexia, and a tendency for the animal to tire easily when made to exert itself. While there was considerable variation in the temperature reaction, as a rule on the 1st day it reached 40.4°C. but seldom exceeded 41°C. On the 2nd day of illness the fever, anorexia, and malaise were more marked. By the 3rd day the temperature had as a rule reached its peak and frequently exceeded 41.5°C. and sometimes even 42°C. At this time respiratory involvement was manifested by increased rate and a peculiar type of diaphragmatic breathing, described popularly as "thumping," and frequently a paroxysmal type of cough was elicited when the animals were roused. They exhibited marked prostration, refused food, and lay listlessly in their pens. Their

TABLE I
Leucocytic Reaction in Experimental Swine Influenza

Swine No.	Before inoculation	Days after inoculation				
	Leucocytes per c.mm.	2	3	4	5	6
		Leucocytes per c.mm.	Leucocytes per c.mm.	Leucocytes per c.mm.	Leucocytes per c.mm.	Leucocytes per c.mm.
459	28,000	24,200	22,000	13,300		
461	19,000	14,000	10,800	12,040		
549	24,400		13,400	14,600	7,400	
555	23,000	17,900	17,300	19,800		21,400
562	28,800	27,600	20,300		11,400	
572	22,600	15,540		11,840		14,200
583	21,920		17,600		15,500	
587	14,940		9,240		13,400	

condition on the 4th and 5th days was little altered from that on the 3rd day. Death occurred on the 3rd, 4th, 5th, or 6th days and was preceded by an exaggeration of all respiratory symptoms, an increase in the prostration, and the onset of an active, incoordinated delirium during which the animal lay on its side and made running motions with its legs or, attempting to stand, would stagger about the isolation unit. On the 6th day as a rule, the temperature was definitely receding in animals that were to recover and from then on recovery was rapid.

In both the natural and the experimental disease a mild leucopenia was usually observed. A record of a few counts on experimental cases is given in Table I. There was no significant difference in the degree of leucopenia of the various cellular types as shown by differential counts.

Pathology

Descriptions of the lesions of swine influenza are fragmentary and deal entirely with fatal cases of the disease.

Dreher (5) has reported the main features as congestion of the mucous membranes of the respiratory tract, a lobular type of pneumonia, swollen and edematous bronchial lymph nodes, and congestion of the gastric mucosa. Dimock and Healy (3) have described the lungs as congested, edematous, fully distended and heavy, and frequently exhibiting bronchopneumonia. Quinn (1) has emphasized the occurrence of extensive edema of the lungs and Murray and Biester (7) found edema to be frequent. The latter authors also called attention to the similarity existing between the "water logged" lung of the human influenzal pneumonia of 1918 and that of the pneumonia of swine influenza.

It has seemed important to study the lesions at various stages of the illness. The large majority of the animals sacrificed would have progressed to an uneventful recovery. The findings in fatal cases will be described separately.

Gross Pathology.—The swine were sacrificed on the 1st to the 5th day of fever. The large majority were chloroformed while a few were stunned by a blow on the head and then bled to death. There were no external signs of disease at the time of slaughter save in some of the animals which seemed most ill. These sometimes exhibited a watery mucous nasal discharge.

The mucosa of the pharynx and larynx was, as a rule, very mildly hyperemic and covered by a white, glassy, tenacious mucus. On opening the trachea the same type of tenacious mucus was present in from moderate to copious amounts. It was sometimes frothy, very rarely blood-tinged. There was more exudate present in the large bronchi and, in the smaller bronchi and bronchioles, it completely filled the lumen. In the bronchioles the exudate was of firmer consistency than higher in the respiratory tract. It not infrequently could be removed in small, white, semitranslucent, sago-like masses. The pleural sacs were free of excess fluid or fibrin. The lungs presented very constant and characteristic gross changes. The involved lung tissue was a deep purplish red in color, noticeably depressed when compared with the uninvolved lung tissue, and the line of demarcation between normal and pathological lung substance was very definite. Palpation of the involved lung revealed that it felt "leathery," did not crepitate, and contained no areas of consolidation. The elements of the bronchial tree could be palpated and gave the impression of being thickened. On cut section the bronchioles protruded from the surface and the lung substance itself had a purplish red, "beefy," pasty appearance throughout. The gross picture was that of a

marked massive atelectasis, irregular both as to amount and distribution. It was usually limited to portions of the cephalic, cardiac, and azygos lobes, and it not infrequently involved all five of these lobes. In over half of the cases it was bilateral and irregularly symmetrical. If, however, it tended to be unilateral, the right side was almost always predominantly involved. There were a number of cases in which only the right cephalic and the cardiac lobe exhibited massive atelectasis. An illustration of a typical case is presented in Figs. 1 and 2.

The adjoining non-atelectatic portions of the lungs were emphysematous. The surfaces of these portions of the lung were elevated and extremely pale. A feature occasionally observed was a moderate to extreme petechiation of the pleurae overlying the areas of compensatory emphysema. In some cases a moderate interlobular edema of the non-atelectatic areas of lung was encountered.

The cervical, mediastinal, and mesenteric lymph nodes were extremely enlarged and very edematous. They were only rarely congested. Those at the hilum of the lung were sometimes so large as to resemble grapes. On section they were found to be soft and to ooze fluid in relatively large amount.

A small excess of fluid was not infrequently encountered in the peritoneum. This was as a rule clear and straw-colored but sometimes slightly cloudy and contained flecks and strands of fibrin. There was usually, but not invariably, a moderate acute splenic tumor. The kidneys and liver were negative in the gross. The stomach was sometimes found to contain a bile-tinged watery mucus and the gastric mucosa was almost invariably extremely hyperemic especially along the greater curvature and at the cardiac end. The small intestine was negative.

The mucosa of the colon frequently exhibited hyperemic patches of various sizes. These areas were mildly edematous and overlaid by a scant catarrhal exudate. They never showed a true fibrinous membrane nor were they eroded. The mucosa was elevated over localized areas of submucous lymphoid hyperplasia.

Histopathology.—

Films of bronchial exudate stained for 24 hours in methylene blue revealed a rather constant and characteristic picture. The predominant cell in the exudate was the polymorphonuclear leucocyte and of these there were many. They were as a rule well preserved and not infrequently contained engulfed organisms, usually small thin bacilli but occasionally larger bacillary forms or cocci. There were moderate numbers of lymphocytes in the exudate and smaller numbers of desquamated epithelial cells. Lying between the cells of the exudate were large numbers of extremely thin, very faintly staining, hair-like structures, evidently broken off cilia. In some preparations these were extremely numerous. Organisms of the type seen in the leucocytes were sometimes very numerous between the cells of the exudate but as a rule they were scarce.

Tracheal sections showed little that appeared abnormal.

Lung sections cut in such a way as to include small bronchi and terminal bronchioles, and including uninvolved and typically diseased lung exhibited the fol-

lowing features. The small bronchi and terminal bronchioles were filled with a polymorphonuclear leucocytic exudate (Figs. 7 and 8). Bacteria were never numerous in this exudate and frequently they were not demonstrable, or were present in such small numbers as to require careful search to find them. They were most numerous at the junction of the exudate and the bronchial epithelium. The cilia lining the smaller bronchi were either entirely gone or badly matted together. The lining epithelium was fragmented, in places partially desquamated, and the cytoplasm of many of the cells appeared vacuolated (Figs. 4 and 5). In the spaces created by the fragmentation of the lining epithelium, leucocytes, singly or in clumps, were sometimes seen (Fig. 5). There was an extensive peribronchial round cell infiltration (Figs. 4, 5, 7, 8). The areas of lung that appeared to be merely atelectatic were found histologically to present other changes than atelectasis alone (Figs. 9 and 10). They were of lobular distribution, and sharply demarcated from adjacent uninvolved lung by interlobular septa, although a number of adjacent lobules might be, and usually were, involved. In these areas the alveoli were collapsed and frequently contained desquamated epithelial cells, small numbers of mononuclear wandering cells, and occasionally some coagulated plasma. Large, feebly stained cells exhibiting a "foamy" cytoplasm were especially numerous in some sections. Leucocytes and red cells were not found regularly in the alveoli although it was difficult to find sections, even from very early cases, in which the alveoli in some areas of the section did not contain leucocytes and occasionally red cells in small numbers. Leucocytes when present were most abundant in the alveoli opening directly into the terminal bronchioles. The alveolar walls were wrinkled and broadened and definitely infiltrated with mononuclear cells (Fig. 6). This infiltration was most marked in the alveolar walls adjacent to the bronchi but it was present and frequently conspicuous throughout the entire area of atelectasis. Even in non-atelectatic areas of lung there sometimes was some slight to moderate distension of the alveolar walls due to a round cell infiltration. There was a moderate passive congestion of the atelectatic areas as evidenced by dilated and injected pulmonary capillaries. The lymph channels, especially in the interlobular septa, were sometimes dilated and filled with lymph and small numbers of cells. They were not observed to be thrombosed or to contain bacteria. The interlobular septa were frequently widened owing not only to the dilation of the lymph channels but to an apparent pulling apart of the connective tissue elements and to some round cell infiltration. The pleura overlying the atelectatic areas was sometimes wrinkled and thrown into small folds (Fig. 10). It was apparently unaltered otherwise. Lobules lying adjacent to areas of atelectasis were markedly emphysematous, exhibiting extremely thin alveolar walls, many of them broken.

It should perhaps be indicated here that even when all five of the upper lobes of the lung of swine are involved, over half of the actual lung substance remains relatively intact, for the diaphragmatic, or lower lobes, are greater in volume than the five upper lobes. Since it is unusual to have complete atelectasis of all five of the upper lobes, in most cases no more than one-third of the total lung volume is actually involved.

Outside the respiratory tract the histopathology was in accordance with the gross pathological picture. The cervical, mediastinal, and mesenteric lymph nodes were as a rule packed with lymphocytes, the germinal follicles appeared active, and there was much intercellular edema. The histological picture presented by the spleen was that of a slight to moderate, acute splenic tumor characterized by a moderate increase in the mononuclear cells in the pulp and very little, if any, alteration in the Malpighian bodies. The liver was sometimes passively congested but otherwise negative. The kidneys were negative. The mucosa and submucosa of both the stomach and colon showed an increase in mononuclear round cells which was sometimes quite extreme. The blood vessels in these regions were usually dilated and packed with red cells. The goblet cells in the mucosa of the colon were prominent and distended.

As this description shows, the pulmonary pathology differs markedly from that encountered at autopsy in animals succumbing to either the experimental or spontaneous disease.

The Pathology of Fatal Cases.—Death in fatal cases was not necessarily due to pathological changes induced by secondary bacterial invaders, for swine influenza of itself can kill.

The postmortem picture presented by swine dead of the experimentally induced disease was the same as in fatal spontaneous cases. The cervical lymph nodes were much enlarged, edematous, and frequently congested. The mucosa of the trachea and larger bronchi was moderately congested and covered with a thick, tenacious, and sometimes frothy mucous exudate. The smaller bronchi contained a more fluid, blood-tinged exudate and this sometimes in copious amounts. A sero-sanguineous pleural exudate was frequently encountered. This exudate sometimes contained considerable fibrin and in these cases the anterior lobes of the lung were covered with closely adherent fibrin. Both pleurae were usually involved, although sometimes the process was unilateral. The lungs themselves were voluminous, heavy, and mottled purplish red in color. Palpation revealed that only the apical, azygos, or cardiac lobes were consolidated. That is, the true pneumonia was limited entirely to the portions of lung which in uncomplicated swine influenza would have been atelectatic. The diaphragmatic lobes, which in swine comprise well over half the actual lung substance, exhibited a hemorrhagic type of pulmonary edema which was in most instances extreme. The markings of the interlobular septa were widened by fluid and the lobes as a whole had a glistening swollen appearance. When they were cut across there was an outpouring of a frothy, bloody fluid. There was, as a rule, no fibrin adherent to the diaphragmatic lobes.

Histological examination of sections of the pneumonic areas revealed the pleurae to be usually overlaid with a rich network of fibrin in the meshes of which were myriads of leucocytes. The bronchioles were completely filled with leucocytes, the lining epithelium was badly fragmented and partially desquamated, and the

bronchial walls were densely infiltrated with round cells. The alveoli throughout the section were filled with leucocytes, red blood cells, and coagulated plasma. There was usually no fibrin in the alveolar exudate. The alveolar walls were mildly folded, thickened, and infiltrated, largely with round cells. The lymph sinuses in the interlobular septa were dilated and contained leucocytes, some lymphocytes, and much lymph.

Histological examination of sections of the edematous portions revealed plasma filling the alveoli which also contained small numbers of desquamated epithelial and red cells. There was no fibrin. The cellular exudate in the bronchi was scant but there was much plasma. The bronchial walls were thickened owing to intercellular edema and some round cell infiltration. The pulmonary capillaries were usually dilated and packed with red cells. The lymph sinuses in the interlobular septa and beneath the pleura were widely dilated and contained, in addition to plasma, small accumulations of leucocytes and lymphocytes. The lymph nodes at the hilum of the lung were always enlarged and edematous and usually moderately congested. In some cases, especially those in which a fibrinous pleuritis had been encountered, a serofibrinous pericarditis was also to be observed. The livers of fatal cases of swine influenza were usually engorged and the spleens large, swollen, dark, and friable. An acute diffuse nephritis was occasionally encountered. The stomachs were usually empty except for the presence of moderate to copious amounts of a thin bile-tinged mucus. The small intestines also usually contained considerable bile-tinged mucus. The mucosa of the cecum and the first two-thirds of the colon was intensely hyperemic.

Animals slaughtered during convalescence have much interest from a pathological standpoint. Pulmonary evidence of the disease persisted for 3 or 4 weeks or longer following clinical recovery. Areas of lung were found that were slightly depressed as compared with the adjoining normal tissue. They were grayish pink, on palpation felt firmer than normal, and cut as though fibrous. The bronchi in some instances were dilated and contained a mucopurulent material. Sections revealed much fibrous tissue and great thickening of the alveolar walls.

In animals convalescent from a swine influenzal pneumonia, lung abscess, bronchiectasis, or an obliterative pleuritis were not infrequently encountered at autopsy.

SUMMARY

Swine influenza has been induced in pigs by the intranasal instillation of material from spontaneous cases of the disease as occurring epizootically in eastern Iowa. The experimental disease has the same

features as the epizootic. It has been maintained for study by serial passages accomplished either by intranasal instillation or by pen contact. Eight strains of the virus have been established experimentally during three epizootic periods. The clinical disease induced by these eight strains has been in general the same although its severity and mortality have varied.

The principal features of the pathology of swine influenza are an exudative bronchitis accompanied by marked damage of the bronchial epithelium and its cilia, a peribronchial round cell infiltration, and massive pulmonary atelectasis. The latter is modified somewhat by a round cell infiltration of the alveolar walls. The lymph nodes, especially the cervical and mediastinal ones, are hyperplastic and edematous. There is usually a mild to moderate, acute splenic tumor. The mucosa of the stomach and colon is congested.

The pneumonia following swine influenza is, characteristically, lobular in type and of the same general distribution as the atelectasis. The non-pneumonic areas of lung are extremely edematous and congested.

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EXPLANATION OF PLATES

PLATE 32

FIG. 1. Dorsal aspect of lung in experimental swine influenza (Swine 895) to show the typical appearance and distribution of the massive pulmonary atelectasis. The lymph nodes at the hilum are swollen and edematous. The sharp demarcation between atelectatic and non-atelectatic lung is noteworthy. Animal chloroformed on the 4th day of illness.

FIG. 2. Ventral aspect of same lung.

PLATE 33

FIG. 3. Section of a small bronchus from a normal hog (Swine 588) showing intact ciliated epithelium overlying a normal submucosa. Animal killed by stunning and bleeding. Eosin-methylene blue. $\times 305$.

FIG. 4. Section of a small bronchus from a spontaneous field case of swine influenza (Swine 13) showing fragmented epithelium and extreme round cell infiltration of the submucosa. Animal stunned and bled. Eosin-methylene blue. $\times 305$.

FIG. 5. Section of a small bronchus in experimental swine influenza (Swine 564) showing leucocytic bronchial exudate, fragmented and vacuolated epithelium denuded of cilia, and round cell infiltration of the submucosa. Leucocytes have invaded the mucosa. Animal chloroformed on 5th day following inoculation. Eosin-methylene blue. $\times 305$.

FIG. 6. Section of lung in experimental swine influenza (Swine 473) showing thickening of the alveolar walls in an area of atelectasis. The cells infiltrating the alveolar walls are largely round cells. Animal chloroformed on 3rd day following inoculation. Eosin-methylene blue. $\times 295$.

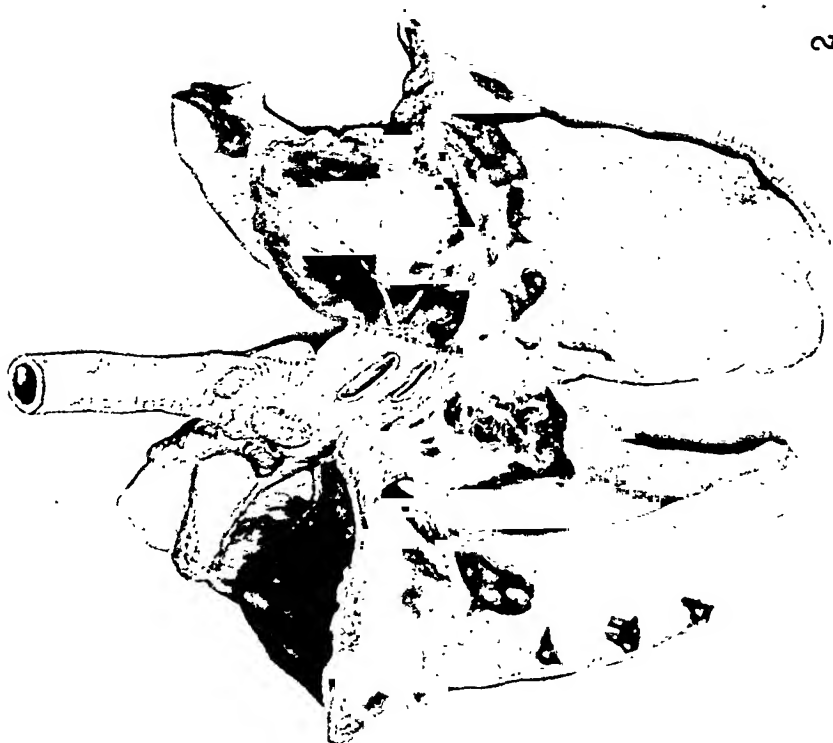
PLATE 34

FIG. 7. Section of lung from a spontaneous field case of swine influenza (Swine 13) showing a bronchus in an area of compensatory emphysema to illustrate better the dense peribronchial round cell infiltration. The lumen of the bronchus contains a dense leucocytic exudate. Animal stunned and bled. Eosin-methylene blue. $\times 51$.

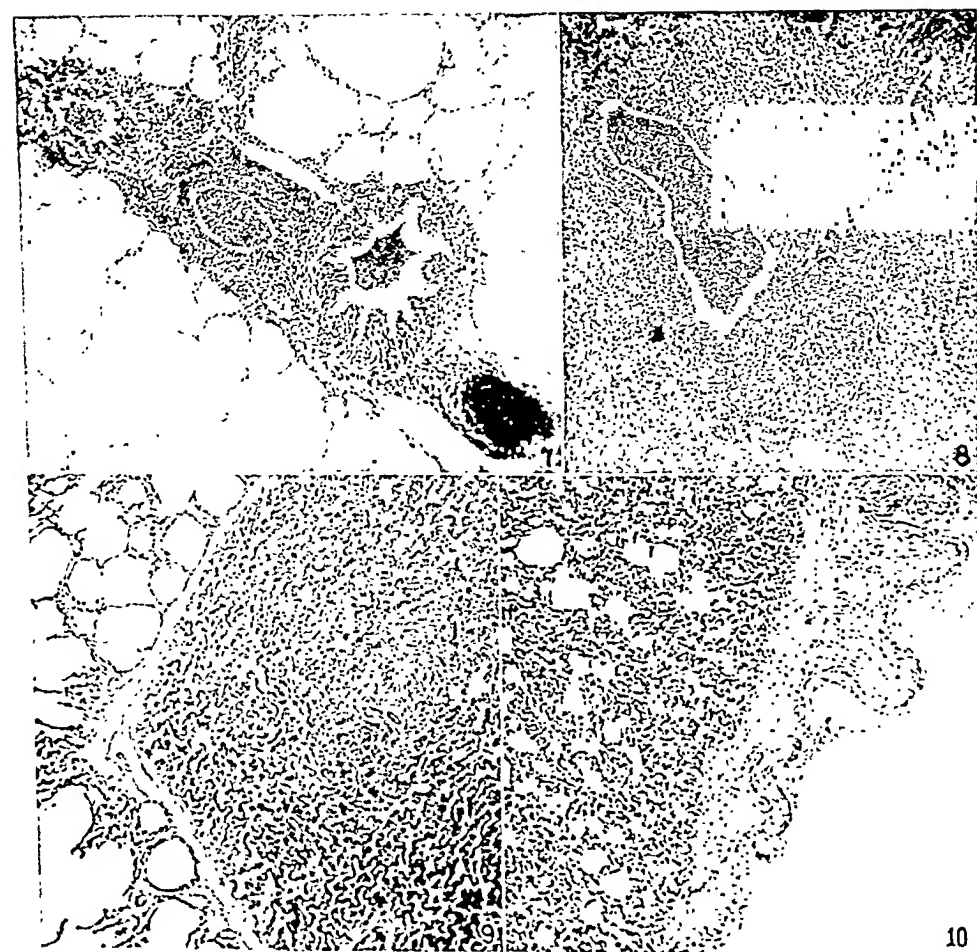
FIG. 8. Section from the lung in experimental swine influenza (Swine 593) showing dense leucocytic exudate in small bronchus and peribronchial infiltration largely with round cells. Animal chloroformed on 6th day following inoculation. Eosin-methylene blue. $\times 51$.

FIG. 9. Section of lung from a spontaneous field case of swine influenza (Swine 97) showing atelectasis with infiltration of the alveolar walls, slight leucocytic exudate in some of the collapsed alveoli, and compensatory emphysema. Animal stunned and bled. Eosin-methylene blue. $\times 51$.

FIG. 10. Section of lung in experimental swine influenza (Swine 454) showing folding of pleura over an area of atelectasis. Animal chloroformed on 3rd day following inoculation. Eosin-methylene blue. $\times 51$.







SWINE INFLUENZA

II. A HEMOPHILIC BACILLUS FROM THE RESPIRATORY TRACT OF INFECTED SWINE

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PLATE 35

(Received for publication, May 6, 1931)

Murray (1), McBryde, Niles, and Moskey (2), and recently Fulton (3) have attempted to discover the nature of the inciting agent of swine influenza. Murray described a small Gram-negative coccus as the cause of the disease. By inoculating normal swine intravenously with relatively small doses of pure cultures of this organism, he induced a disease which he believed was clinically and pathologically swine influenza. McBryde, Niles, and Moskey failed to obtain Murray's micrococcus in their cultures. They isolated only two types of organisms from cases of the disease with any degree of frequency, a pleomorphic Gram-positive bacillus and *Bacillus suisceplicus*. Neither of these organisms produced the disease when injected into susceptible swine. Fulton recently confirmed the presence of McBryde's pleomorphic Gram-positive bacillus in the respiratory tracts of swine with influenza and, with very freshly isolated cultures, reproduced the disease experimentally. However, cultures that had been transferred only three times or original cultures that had been kept on ice for as short a period as 2 weeks were incapable of inducing influenza when administered intranasally to normal swine.

Spray (4) using slaughter house material cultured the respiratory tracts of a large number of normal swine and of those showing pneumonia. From both classes he frequently isolated an inulin-fermenting streptococcus and *B. suisceplicus*. These organisms were encountered more often from pneumonic than from normal lungs; the inulin-fermenting streptococcus was frequently present in pure culture from the pneumonic areas. Since Spray did not differentiate infectious pneumonia, hemorrhagic septicemia, and influenza, his findings are not of great value.

EXPERIMENTAL

Bacteriological studies have been conducted upon a large number of swine experimentally infected with swine influenza from the 1928, 1929, and 1930 epizootics (5), and upon a small number of spontaneous field cases of the disease from the mild 1929 epizootic. In addition,

thirty-five swine not suffering from influenza have served as a control group. These animals were either normal, or infected with hog cholera or spontaneous pneumonia. They were from local sources similar to those of the animals used in the study of experimental swine influenza.

In suspected cases of influenza, primary cultures were made from atelectatic or pneumonic lung, bronchial exudate, spleen, and, in certain cases where indicated, from other organs, on plain agar slants (pH 7.3 to 7.6), to the condensation fluid of which 0.5 to 1 cc. of sterile defibrinated horse or swine blood had been added. Cultures of heart blood were made on plain agar slants inoculated by pipette with approximately 1 cc. of blood. In some cases primary cultures were also made on blood and chocolate agar plates. Similar cultures were made from the control animals on the same kinds of media.

The suspected organism was very similar to, if not identical with, *H. influenzae*. The first culture was obtained from the heart blood of our first experimental case of the disease. This blood was apparently sterile when streaked on plain and blood agar plates. In approximately 1 cc. amounts it was added to the condensation fluid of about twenty plain agar slants which were incubated for 2 days. At this time the slants appeared sterile. The blood was not hemolyzed and there was no evidence of growth. However, films prepared from the bloody condensation fluid of these slants, when stained with methylene blue and examined microscopically, revealed the presence of organisms in moderate numbers. These were predominantly thin, curved bacilli which varied considerably in length. Some were less than 1μ long while other apparently single individuals sometimes approached 4μ in length. Some threads of continuous protoplasm extended completely across the field. Other shorter threads appeared as tangled masses enmeshing small curved bacillary forms and both large and small coccoidal forms. Attempts to obtain subcultures on plain agar, infused blood agar, inspissated serum, or in bouillon or Smith-Noguchi medium were unsuccessful. Growth could be continued, however, in sterile defibrinated swine or horse blood at the bases of plain agar slants and fair growth reaching a maximum in 48 hours occurred on chocolate agar.

This organism has been found in the respiratory tracts of all swine experimentally infected with influenza that have come to autopsy within 7 days of the onset of fever; it has been recovered from the respiratory tracts of six spontaneous cases of the disease from five different herds (all of the spontaneous cases which were examined); and it has been found in the original material used in establishing the eight strains of the disease which have been studied experimentally (5). In the case of all eight strains the organism has been recovered from the respiratory tract of the first experimentally infected animal

TABLE I

The Incidence of H. influenzae (Variety suis) in Experimental and Spontaneous Swine Influenza

Strain	No. of swine cultured	Material cultured	<i>H. influenzae suis</i>				
			Present	Absent	Per cent positive	Present in pure culture	Per cent present in pure culture
1 (1928)	33	Lung	28	4	87	11	34
		Bronchial exudate	17	6	74	8	35
		Heart blood	6	20	23	5	19
2 (1928)	9	Lung	7	2	78	4	44
		Bronchial exudate	8	1	89	3	33
		Heart blood	0	9	0	0	0
5, 6, 7, and 10 (1929) combined	7	Lung	7	0	100	5	71
		Bronchial exudate	6	0	100	2	33
		Heart blood	0	7	0	0	0
Field cases (1929)	6	Lung	5	1	83	1	17
		Bronchial exudate	6	0	100	2	33
		Heart blood	2	4	33	0	0
14 (1930)	8	Lung	6	2	75	5	63
		Bronchial exudate	8	0	100	2	25
		Heart blood	2	5	29	2	29
15 (1930)	25	Lung	23	2	92	15	60
		Bronchial exudate	24	0	100	9	38
		Heart blood	8	17	32	8	32

TABLE II

The Incidence of Hemophilus influenzae (Variety suis) in a Control Group of Animals

Group	Material cultured	<i>H. influenzae suis</i>		
		Present	Absent	Percentage
Swine experimentally infected with hog cholera—16	Lung	0	16	0
	Bronchial mucus	0	13	0
Normal swine—8	Lung	0	8	0
	Bronchial mucus	0	8	0
Swine dying of or showing pneumonia at autopsy—11	Lung	0	11	0
	Bronchial mucus	0	8	0
Iowa swine suffering from a non-influenzal respiratory disease—4	Lung	0	4	0
	Bronchial mucus	0	4	0

in each series. In these groups of cases it has frequently been present in pure culture. Because of its growth requirements, porcine origin, and morphological and cultural characteristics, to be discussed later, the name *Hemophilus influenzae* (variety *suis*) is suggested for this organism. It will be referred to hereafter as *H. influenzae suis*. The incidence of *H. influenzae suis* in influenza-sick swine killed within 7 days after inoculation is recorded in Table I. In addition to the cultures recorded in Table I, it has been recovered from the pleura or pericardium whenever these have contained exudate, and from the spleens of most fatal cases. In two cases which developed otitis media during the course of their experimental disease it was the predominant organism in the middle ear exudate. It has not been encountered in the swine serving as bacteriological controls, as indicated in Table II.

Cultural and Morphological Characters of H. influenzae suis

Morphologically and in the character of its growth on media *H. influenzae suis* resembles *H. influenzae* very closely.

Media to sustain growth must contain both factors V and X (6). Hematin prepared by the method of Anson and Mirsky (7) and supplied by them was used as the source of factor X, while factor V was furnished either by an actively growing culture of a non-hemolytic streptococcus (spotted on the hematin-containing plate) or by a sterile Berkefeld filtrate of an old bouillon culture of this streptococcus added to hematin-containing agar.

Chocolate agar is the best solid medium and heated blood bouillon the most satisfactory fluid medium for the growth of *H. influenzae suis*. Cultures of the organism that have been well established grow feebly and as very minute colonies on blood agar. In mixed culture on blood agar luxuriant growth of *H. influenzae suis* colonies occurs about the colonies of the contaminating organism. On chocolate agar well established cultures reach a maximum in between 24 and 48 hours at 37°C. The colonies on this medium vary from less than 1 mm. to more than 2 mm. in diameter, are circular, grayish, semitranslucent, flattened, and have a sharply contoured edge. Films prepared from chocolate agar cultures contain three distinct forms. Examined after 24 hours' growth the predominant form is a small thin bacillus varying from less than 0.5μ to 2μ in length and approximately 0.2μ in thickness (Fig. 1). A few longer, curved, thread-like forms appear also. 48 hour cultures contain more of the long thread-like types and in some cultures these form clumps of tangled masses of organisms (Fig. 2). Small coccoidal organisms are found at 48 hours. Cultures examined at 3 days and thereafter reveal

increasing numbers of the coccoidal forms. In blood bouillon or heated blood bouillon 24 hour cultures are found to be composed largely of small, thin, straight or slightly curved rods. A few coccoidal forms are present and these usually in clumps of from six to thirty or more organisms, seldom singly. At 48 hours almost the entire culture is composed of clumps of small cocci (Fig. 3). In fluid media the long thread-like forms are not regularly encountered. "Giant" coccoidal and large club and comma shaped forms are numerous in some cultures (Fig. 4).

The capacity of films of cultures from either solid or fluid media to absorb the usual dyes decreases rapidly with the age of the culture. Loeffler's alkaline methylene blue, which is the most satisfactory, imparts no more than a faint bluish gray color to organisms from cultures 3 or more days old. By phenolizing the methylene blue and steaming it on the film a slightly more intense color is obtained.

The organism is relatively inert as to growth and fails to act on various substances used for differentiating bacteria. In blood bouillon, with brom-cresol purple as indicator, definite growth occurs. There is, however, no demonstrable action on dextrose, lactose, saccharose, dulcitol, mannitol, glycerol, inulin, or arabinose. Some fading of the indicator occurred in all tubes. All cultures were observed for 7 days. None of the cultures examined produced indol or hydrogen sulfid. Litmus milk containing blood fostered but slight growth and was unchanged. Nitrates were reduced to nitrites by all cultures examined. No growth could be obtained with well established cultures on plain or glycerin agar, Dorset's egg medium, potato, coagulated blood serum, or in gelatin. The organism is non-hemolytic, non-motile, and Gram-negative.

As may be evident from the preceding description, there is no real basis for differentiation between *H. influenzae suis* and non-indol-producing strains of *H. influenzae*. If a culture of *H. influenzae suis* were isolated from a human throat, no doubt would be raised as to its identity with *H. influenzae*. Since primary cultures of *H. influenzae suis* grow somewhat more feebly on heated blood agar than do primary cultures of *H. influenzae*, such a hypothetical culture would perhaps be considered more difficult to establish than the typical Pfeiffer bacillus.

For the primary isolation of *H. influenzae suis*, the plain agar slant containing from 0.5 to 1 cc. of sterile defibrinated horse blood at the base is the medium of choice. In this medium the organism grows in the blood at the base of the slant and the pure culture on inspection appears sterile. Examination of stained films of the bloody condensation fluid, however, readily reveals the organism in characteristic form. It is felt that for the isolation of *H. influenzae suis* this medium is superior to heated blood agar. It has been the practice not to examine

TABLE III
Direct Agglutination

Culture strains	Agglutinating sera														<i>H. influenzae</i> BIW																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												
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	14	451	459	660											1:50	1:100	1:200	1:400	1:600	1:800	1:1200	Control																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
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* C = complete agglutination; +, strong agglutination; x, weak to trace agglutination.

primary cultures until after 48 hours' incubation, since this length of time is required for the development of the curved and tangled thread-like forms which are characteristic and identifiable even in mixed culture. Cultures are viable in this medium for 2 weeks, but stock cultures have been transferred at weekly intervals and kept in the incubator.

Serological Relationship between Strains of H. influenzae suis and of H. influenzae

It was realized that, because of the great serological diversity of strains of *H. influenzae*, attempts to demonstrate any serological relationship between *H. influenzae suis* and *H. influenzae* would be inconclusive. However, the near identity of the two organisms both culturally and morphologically made it seem of interest to compare the reciprocal agglutinability of a few strains of each organism. The cultures of *H. influenzae* and the agglutinating serum were generously provided by Dr. Olga R. Povitzky of the laboratories of the New York Department of Health. The *H. influenzae* serum was of very high titer whereas the sera prepared with cultures of *H. influenzae suis* were of relatively low titers. All agglutination tests were conducted at 55°C. and readings were made after 2 hours at this temperature and refrigeration overnight. Cultures of *H. influenzae suis* tested were from widely separated sources and no two cultures were from the same strain of the disease (5). The results of these direct agglutination tests are given in Table III.

The data given in Table III indicate only one possibility that any two of the *H. influenzae suis* cultures are serologically identical. By use of the agglutinin absorption test it was possible to demonstrate that Cultures 652 and 660 were serologically identical. Conversely, by means of the agglutinin absorption test, it was possible to demonstrate that cultures of *H. influenzae suis* 451, 459, 652, and 660 which were agglutinated, but not to titer, by *H. influenzae* BIW were capable of removing no agglutinin for *H. influenzae* BIW.

Pathogenicity of H. influenzae suis for Laboratory Animals

As a rule *H. influenzae suis* is non-pathogenic for rabbits and can be administered intravenously to them in doses of 2 cc. of a heavy suspen-

sion of growth from chocolate agar without inducing any observable evidence of illness. However, such injections occasionally prove fatal in from 3 to 6 hours after injection.

One culture, when injected intraperitoneally, has been found to kill

TABLE IV

The Effect of Inoculating Swine with Pure Cultures of H. influenzae suis

Swine No.	Route of inoculation	Culture used			Clinical evidence of illness	Autopsy findings
		No.	Time since isolation	No. of transfers on media		
507	i.n.*	451	18 days	4	Suggestive of influenza	Suggestive of influenza
515	i.v.**	451	18 "	4	Moribund in 3 days	No respiratory pathology
739	"	451	17 mos.	86	Negative aside from transient temperature elevation	Not autopsied
591	i.n.	Mixture of 1928 cultures	All over 3 mos.	All 15 or more	Negative	Negative
590	"	"	All over 2 mos.	All 10 or more	"	"
588	"	11-T	12 days	6	"	"
648	"	15	16 "	5	"	"
651	"	660	17 "	5	"	"
826	"	791 + 794	14 and 17 days	7 and 8	Death in 2 days	Pneumonia
873	"	451 + 459	Over 2 yrs.	125 and 113	Negative	Negative
893	"	451 + 459	" 2 "	126 and 114	"	"
904	"	896	9 days	7	"	"
912	"	451	Over 2 yrs.	131	"	"
917	"	905	16 days	7	"	"
922	"	451	Over 2 yrs.	132	"	"

* i.n. = intranasally.

** i.v. = intravenously.

guinea pigs in from 18 to 24 hours. Two other cultures are completely non-pathogenic for guinea pigs.

About 50 per cent of the white mice injected intraperitoneally with various strains of *H. influenzae suis* have died. The organism is non-pathogenic when injected intraperitoneally into white rats.

Pathogenicity of H. influenzae suis for Swine

In an attempt to determine the etiological relationship of the organism to swine influenza, thirteen swine were inoculated intranasally with cultures of *H. influenzae suis* from various sources and under cultivation for varying periods of time on artificial media. Two swine received the organism intravenously. The cultures used in these experiments were grown in defibrinated blood at the bases of plain agar slants and the usual dose administered was 2 cc. of this bloody condensation fluid. The results of these experiments are recorded in Table IV.

The data presented in Table IV indicate that *H. influenzae suis* was completely non-pathogenic for eleven of the thirteen swine to which it was administered intranasally. Given intravenously, a relatively recently isolated culture induced a severe illness. However, at autopsy no disease of the respiratory tract suggestive of swine influenza was encountered. There was an acute glomerular nephritis. An older culture given intravenously to another animal proved non-pathogenic.

Swine 507 and 826 are noteworthy in that both animals became ill after intranasal inoculation with *H. influenzae suis* and, in both, the picture presented could readily have been confused with swine influenza. Swine 507 was infected during the early part of the 1st year's work with the disease and at the time was considered to be a fairly typical although mild case of swine influenza. Since *H. influenzae suis* was obtained in pure primary culture from both the bronchial exudate and lung of this animal at autopsy, Koch's postulates had apparently been fulfilled for the organism, and the writers at first believed that it was the inciting agent of the disease. Subsequent work has indicated the mistaken nature of this view but no certain and acceptable explanation of this one experiment, provided Swine 507 actually had influenza, can be offered. It may be that the four transfers on artificial media to which the organism was subjected before its use in inoculating Swine 507 were insufficient to remove mechanically any accompanying virus. Swine 826 died 2 days after inoculation with *H. influenzae suis* and at autopsy presented pulmonary lesions very like those observed in fatal cases of swine influenza. The true pneumonia was limited to the cephalic lobes and, as in fatal swine influenza, the caudal lobes presented an intense bloody edema. *H. influenzae suis* was recovered in pure culture from the kidney, heart blood, and spleen, and was obtained in mixed culture from the lung and bronchial exudate. The disease which developed in Swine 826, however, was recognized as definitely different from swine influenza in that it was not contagious. A normal susceptible

hog placed in the same unit soon after Swine 826 had been inoculated developed no disease. This is markedly inconsistent with the highly contagious nature of swine influenza. A possible explanation of the fatal pneumonia in Swine 826 is that this animal had been taken from its mother at birth, deprived of colostrum, and fed an artificial diet. The influence of such treatment on the course of an influenzal infection will be discussed more fully later.

The experiments recorded in Table IV seem sufficient to demonstrate that *H. influenzae suis* alone is not capable of inducing swine influenza even though it may under certain conditions be pathogenic for swine.

SUMMARY

1. A hemophilic bacillus has been regularly obtained in culture from the respiratory tract of a series of swine experimentally infected with swine influenza and from a small number of spontaneous field cases of the disease. It has not been observed in respiratory tract cultures from a group of swine free from influenza.

2. The cultural and morphological characters of the organism have been described and the name *Hemophilus influenzae* (variety *suis*) suggested. The organism exhibits marked serological diversity, since only two out of eight strains studied were serologically identical. It is usually non-pathogenic for rabbits and white rats, and irregularly pathogenic for white mice. One strain of the organism was pathogenic for guinea pigs while two others were not.

3. Eleven out of thirteen attempts to induce symptoms of disease in swine by intranasal inoculation with pure cultures of *H. influenzae suis* were entirely negative. The remaining two attempts which suggested a positive result have been discussed.

4. Attention has been called to the marked similarity which exists between non-indol-producing strains of *H. influenzae* and *H. influenzae suis*.

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EXPLANATION OF PLATE 35

Films from cultures of *H. influenzae suis*. $\times 905$.

FIG. 1. 24 hour chocolate agar culture of Strain 660 showing predominantly small bacillary forms. Alkaline methylene blue.

FIG. 2. 48 hour chocolate agar culture of Strain 913 showing predominantly long thread-like forms. Dilute carbol-fuchsin.

FIG. 3. 72 hour blood broth culture of Strain 913 showing clumps of small coccoidal and bacillary forms. Dilute carbol-fuchsin.

FIG. 4. 5 day blood broth culture of Strain 459 showing clumps of "giant" coccoidal and club-shaped forms. Alkaline methylene blue.



SWINE INFLUENZA

III. FILTRATION EXPERIMENTS AND ETIOLOGY

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McBryde, Niles, and Moskey (1) made five attempts to pass the etiological agent of swine influenza through small Berkefeld or Mandler filters but were unable to reproduce the disease by dropping such filtrates into the nostrils of normal hogs. In two of these experiments unfiltered material failed to produce the disease in their control animals. Although these experiments are too few in number to be conclusive, they indicate that the causative agent of swine influenza is not a filtrable virus.

EXPERIMENTAL

Since the studies in filtration reported by McBryde and his coworkers were not conclusive, the question of the filtrability of the etiological agent of swine influenza has been reconsidered. The results of ten filtration experiments with infectious material from the two strains of the disease obtained in 1928 and the two secured in 1930 (2) were inconstant and confusing. In these preliminary experiments the writer was not then cognizant of the possible etiological relationship existing between *H. influenzae suis* (3) and a filtrable agent to be described.

Material for filtration was prepared as follows.

Diseased lung and bronchial lymph nodes were minced with sterile scissors and added to bronchial exudate. This mixture was ground with sand in a mortar. When it had been reduced to a pasty and fairly homogeneous consistency, a 10 to 20 per cent suspension was made by gradually adding sterile distilled water or infusion broth (pH 7.3). It was then shaken with glass beads in a flask for 10 to 15 minutes and centrifuged. The supernatant fluid was removed by pipette and if more than moderately turbid it was centrifuged again. 24 hour bouillon cultures

of *B. prodigiosus* were used to test the efficiency of all filters, and filtrates cultured in 1 cc. amounts on plain agar slants containing defibrinated blood were incubated at 37°C. for 48 to 72 hours before examination for growth.

Swine receiving filtered material were placed in carefully sterilized isolation units where isolation precautions were taken. In certain experiments the control animal receiving unfiltered material and those receiving sterile filtrates were from the same source or even from the same litter.

Of the ten preliminary filtration experiments, three were at the time interpreted as negative, while in the remaining seven some evidence was obtained that the injected filtrate had contained an infectious agent. The disease induced by this filtrable infectious agent, however, was definitely not typical swine influenza and will be referred to hereafter as "filtrate disease."

Clinically the filtrate disease was much milder than swine influenza. In most cases there was no elevation in temperature, while in a few a fever temperature for 1 day was observed. This was at marked variance with the 4 to 6 day fevers seen in typical swine influenza. The usual symptoms shown by filtrate-inoculated swine were a moderate and transient apathy, some diminution in appetite for a period not exceeding 3 days, occasionally a slight cough, and, as in typical swine influenza (2), a moderate or quite marked leucopenia. The extreme prostration so common in swine influenzal infections was not seen. In some instances the disease was so mild that it almost escaped recognition altogether. On this account and in the light of experiments to be outlined later in this paper, it seems possible that in the three preliminary experiments considered as negative, infections were actually produced but so mild in character that they escaped recognition.

The lesions exhibited at autopsy were similar in kind but different in extent, as a rule, from those encountered in typical uncomplicated swine influenza (2). The cervical and bronchial lymph nodes were moderately enlarged and edematous and there was usually a scant to moderate amount of thick, tenacious mucous exudate in some of the smaller bronchi. The amount of pulmonary atelectasis exhibited by filtrate-infected swine varied from a scant amount in one or two of the upper lobes of the lung to an amount as extensive as that shown by mild cases of swine influenza. In all cases, however, the clinical picture was that of the filtrate disease.

It is significant that the filtrate disease was highly contagious and that the incubation period, like that of swine influenza, was about 4 days. In animals infected by contact with filtrate-infected swine the disease was clinically and pathologically identical to that induced by direct inoculation with the filtrate. In experiments to test the con-

tagiousness of the filtrate disease, the normal animals were placed in the pens with inoculated swine after the appearance of symptoms of disease. This was usually 2 days following inoculation.

The filtration experiments just outlined indicated that infectious material from experimental cases of swine influenza contains an agent capable of passage through Berkefeld filters V and N and possessing pathogenic properties when administered intranasally.

H. influenzae suis, which was constantly encountered in culturing the respiratory tracts of animals with typical swine influenzal infections (3), was not found in similar cultures from animals with the mild filtrate disease.

Anaerobic cultures of seven filtrates of swine influenza infectious material in blood broth and in 5 per cent serum bouillon over sterile rabbit kidney have failed to show growth. Four of the seven filtrates thus cultured were tested by intranasal inoculation into swine and all were found capable of inducing the mild filtrate disease.

Intranasal Inoculations with Mixtures of the Filtrable Agent and H. influenzae suis

Since the only constant difference bacteriologically between the mild disease induced by the filtrable agent and typical spontaneous or experimental swine influenza lies in the absence of *H. influenzae suis* in the filtrate-infected swine, the combination of the organism and the filtrable agent may be essential for the production of the natural disease. Experiments were conducted in which swine were inoculated intranasally with cultures of *H. influenzae suis*, which had been under cultivation for a long time (over 2 years in most instances), mixed with Berkefeld filtrates of infectious material from experimental cases of swine influenza.

In these experiments the isolation and filtration practice outlined above was followed. The cultures of *H. influenzae suis* used were grown in defibrinated horse blood at the bases of plain agar slants in most instances for 24 hours. The undiluted blood culture was used in the inoculations and in all experiments the culture injected alone was identical with that mixed with filtrate before injection. The Berkefeld filtrate mixed with cultures of *H. influenzae suis* was identical with that injected alone in individual experiments. With the excep-

Effect of Inoculating Swine with Mixtures of

Experiment No.	Infectious material from swine No.	Swine inoculated No.	Inoculated intranasally with	Clinical picture
1	860 Strain 14 (1930) In infusion broth	859	10 cc. Berkefeld N filtrate	Mild filtrate disease
		861	8 " " " " + 2 cc. culture HIS*	Typical and severe influenza
		871	10 cc. unfiltered suspension	Typical influenza
2	872 Strain 15 (1930) In infusion broth	875	4 " Berkefeld N filtrate	Mild filtrate disease
		874	4 " " " " + 2.5 cc. culture HIS	Typical influenza
		873	2.5 cc. culture HIS in 4 cc. infusion broth	No illness
		876	4 " unfiltered suspension	Typical influenza
3	878 Strain 15 (1930) In distilled water	894	7 " Berkefeld N filtrate + 2 cc. sterile horse blood	Mild filtrate disease
		897	Infected by contact with Swine 894	" " "
		892	7 cc. Berkefeld N filtrate + 2 cc. culture HIS	Typical influenza
		896	Infected by contact with Swine 892	Very severe influenza
		893	2 cc. culture HIS in 7 cc. distilled water	No illness
		895	5 " unfiltered suspension mixed with 10 cc. normal swine serum	Typical influenza
4	907 Strain 15 (1930) In infusion broth	911	4 cc. Berkefeld N filtrate	Mild filtrate disease
		910	4 " " " " + 2 cc. culture HIS	" influenza
		915	Infected by contact with Swine 910	" "
		912	2 cc. culture HIS in 4 cc. infusion broth	No illness
		913	4 " unfiltered suspension	Mild influenza
5	918 Strain 15 (1930) In infusion broth	919	8 " Berkefeld N filtrate**	" filtrate disease
		920	8 " " " "	" " "
		921	8 " " " "	" " "
		923	8 " " " " + 2 cc. culture HIS	Typical and severe influenza
		922	2 cc. culture HIS in 8 cc. infusion broth	No illness
		951	8 " unfiltered suspension	Typical influenza

* HIS = *H. influenzae suis*.** *B. prodigiosus* present in filtrate. *H. influenzae suis*, however, could not be demonstrated. A

Survivable Agent and H. influenzae suis

Autopsy findings	<i>H. influenzae suis</i> in		Remarks
	Lung	Bronchial exudate or scrapings	
by few influenzal pneumonia	Absent Pure culture	Absent Pure culture	Illness extremely mild More severe disease than control (871)
ical	" "	Mixed "	
by few ical	Absent Pure culture	Absent Mixed culture	Control of unfiltered suspension
gative ical	Absent Mixed culture	" " Pure "	Illness extremely mild Disease about same severity as control (87)
autopsied ical	Absent	Absent	Control of culture alone " " unfiltered suspension
influenzal pneumonia tive ical	Mixed culture	Pure culture	Scarcely recognizable illness
	Pure "	Mixed "	" " "
	Absent	" "	Same severity as disease in control (895)
	Mixed culture	Not cultured	Moribund when killed Control of culture alone " " unfiltered suspension
il	Sterile "	Sterile Mixed culture	Scarcely recognizable illness
ve l but few	Pure culture	" "	Same type of disease as control (913)
opsied	Absent	Pure "	" " " " " " (913)
"	"	" "	Control of culture alone " " unfiltered suspension
psied	Pure culture	" "	Scarcely recognizable illness " " "
	" "	" "	" " "
			More severe than disease of control (951)
			Control of culture alone " " unfiltered suspension

rates recorded were sterile.

of 10 days was injected intranasally with 10 cc. of Strain 15 material. After a slightly prolonged latent period the animal developed typical swine influenza with a temperature reaching 41.5°C. At autopsy the disease was typical and extensive.

In this instance, then, serum of a hog that had undergone a typical swine influenza and that was demonstrably immune to reinfection by contact neutralized swine influenza material. No demonstrable immunity developed in the animal receiving the neutral mixture.

Storage of Infectious Material

Experiments to test the keeping qualities of the agents of swine influenza have been complicated by differences in the period of survival of the two components. Pieces of atelectatic lung and bronchial lymph nodes from one experimentally infected swine were stored for 15 to 33 days and from another swine for 15 and 41 days in 50 per cent glycerol before testing them for infectivity. They have been found capable of inducing only the mild filtrate disease typical in its course and at autopsy. With one exception *H. influenzae suis* has not been demonstrable in cultures from the respiratory tract of swine infected with this material. With infectious material frozen and dried by Swift's method (5) the disease induced by stored material was somewhat different. Material that had been stored for 34 days proved, capable of inducing only the filtrate type of disease when inoculated into two susceptible swine and *H. influenzae suis* was not found in the respiratory tracts of these two animals at autopsy. However, another tube of this same material tested after 54 days' storage proved capable of inducing typical and rather severe swine influenza in which at autopsy *H. influenzae suis* was found in both the bronchial exudate and the atelectatic lung. It appears that the swine influenza virus is capable of storage in a dried state or in glycerol for at least 54 or 41 days, respectively, but that the bacterial component of the mixture is less resistant to such storage. The irregularity in the results obtained with dried infectious material may have been due to faulty freezing or drying of the particular tubes of dried material tested after 34 days' storage, for it is difficult to understand why *H. influenzae suis* should not survive freezing and drying. If it were desirable to preserve both factors the virus could be maintained in a dried state or in glycerol, while the organism could be kept under cultivation on artificial media and the two mixed before inoculation.

DISCUSSION

In a series of preliminary experiments to determine the pathogenic properties of bacteriologically sterile Berkefeld filtrates of infectious material from experimental cases of swine influenza, it has been possible in most cases to induce a definite but mild illness by the intranasal inoculation of swine with such filtrates. The disease thus induced has been transmissible by contact without altering its clinical or pathological characteristics. The possibility was thus eliminated that the filtrate disease is the result of inoculation with a toxic bacterial or tissue end-product or aggressin incapable of self-propagation in series. The observation that the illness which developed in animals exposed to cases of the filtrate disease does not differ in its clinical or pathological characteristics from that resulting from direct inoculation with the filtrable agent indicates that the mildness of the filtrate disease is not due to dilution of the inciting agent during filtration. Since the filtrate-induced disease has consistently been at variance with typical swine influenza, it was obvious that the disease induced by a filter-passing virus could not rightly be considered swine influenza. The impression gained after consideration of a series of these mild infections was that the disease both clinically and pathologically represented natural swine influenza in an incomplete form.

In the preceding paper (3) it was shown that a hemophilic bacillus, *H. influenzae suis*, was constantly demonstrable in the respiratory tracts of swine ill with influenza. It has been consistently absent from the respiratory tracts of swine ill with the filtrate disease. To test the possibility that swine influenza is the result of the two agents acting together, swine were inoculated intranasally with mixtures of the filtrable agent and *H. influenzae suis*. A disease typical of swine influenza in all clinical and pathological respects and indistinguishable from that induced by unfiltered infectious material resulted in all instances. Control animals receiving cultures of *H. influenzae suis* alone developed no evidence of illness and swine receiving the filtrable agent alone developed the mild filtrate disease. It seems permissible to interpret these experiments as indicating that swine influenza is due to a filtrable virus and *H. influenzae suis* acting together. Their mode of action is unknown although two possibilities are obvious:

The first possibility is that the pathological activities of the virus are such as to create a portal of entry for *H. influenzae suis* and to furnish a favorable medium in which it can multiply. Under such an assumption the virus serves merely as an entering wedge for the organism and the latter determines the clinical picture and pathology. There can be little doubt from the data presented in this and the preceding paper (3) that, in the presence of the swine influenza virus, *H. influenzae suis* possesses invasive powers which it completely lacks when administered alone.

The second possibility is that the virus is the important component in contributing to the pathology and perhaps also to the symptoms characterizing the clinical picture, and that *H. influenzae suis* increases to a marked degree the pathogenic properties of the virus and hence the severity of the resulting disease. In this respect, the influence of *H. influenzae suis* on the pathogenic properties of the swine influenza virus suggests the effect of certain tissue extracts on various viruses pointed out first by Duran-Reynals (6) and later amplified by Hoffman (7).

Whatever the relation of the virus and the organism in respect to the disease, the data presented indicate that they act together.

The hypothesis is not new that a disease may be induced by a bacterium and an invisible agent, not readily demonstrable alone. It applies most directly to diseases in which the suspected bacterial agent, while readily and uniformly isolated from cases of the disease, either is completely incapable of reproducing the infection or very rapidly loses its ability to do so under conditions of artificial cultivation. It is possible that such organisms do not become non-pathogenic because of rapid loss of virulence but because of the absence of an invisible inciting agent. The proven association of organisms in swine influenza furnishes a tangible and experimentally reproducible justification of this hypothesis.

A question which naturally arises is whether any organism other than *H. influenzae suis* can induce swine influenza in conjunction with the virus. In twenty-one infections of swine with virus alone none of the organisms comprising the normal bacterial flora of the respiratory tract have been capable of producing swine influenza. Also, the constant presence of *H. influenzae suis* in experimental infections

induced by eight strains of swine influenza collected during three separate epizootics would seem sufficient to indicate that, if it is not the only organism capable of completing the etiological complex, it is at least the predominating one for the region from which our original material has been obtained.

It has been of interest to find in several of the fatal influenzal pneumonias that no organism other than *H. influenzae suis* could be cultivated from the pneumonic lungs, heart blood, or pleural and pericardial exudates. It is believed that in these cases, probably because of factors in the host itself, *H. influenzae suis* alone, or in conjunction with the virus, was endowed with invasive and pathogenic characters which in ordinary non-fatal influenzal infections it did not possess.

Since swine are subject to another filtrable virus disease, hog cholera, it has been essential to ascertain that the causative agent of swine influenza is not the hog cholera virus in a disguised or uncommon form. Our evidence indicating that the swine influenza virus is distinct from that of hog cholera may be summarized briefly as follows. Immunization against hog cholera affords no protection against swine influenza, while conversely animals recovered from swine influenza are still susceptible to hog cholera. The administration of hog cholera virus by way of the respiratory tract results in typical hog cholera with no tendency to produce pulmonary lesions suggestive of swine influenza. Convalescent swine influenza serum capable of completely neutralizing swine influenza virus does not neutralize hog cholera virus.

The clinical picture of swine influenza, characterized by fever, anorexia, extreme prostration, leucopenia, and evidence of respiratory involvement and of muscular tenderness, is strikingly suggestive of human epidemic influenza. The onset is sudden, the course short, and convalescence usually uneventful. Death, when it occurs, is the result of an edematous type of pneumonia. The pathology of non-fatal swine influenza, characterized as it is by an exudative bronchitis and extensive pulmonary atelectasis, cannot be compared with the findings in non-fatal human influenzal infections because of our lack of knowledge of the latter. Probably the most significant similarity concerns the predominant bacterium encountered in the two conditions; *H. influenzae suis* is indistinguishable morphologically and culturally from *H. influenzae*. The frequency with which *H. influenzae*

has been encountered in careful bacteriological studies of human influenza parallels the frequency of occurrence of *H. influenzae suis* in swine influenza, and, as in the case of the latter organism, has suggested an etiological significance. Without drawing analogy too far, the irregularity in the outcome of the filtration experiments reported, especially by French and English investigators, in attempting to determine whether a filtrable virus causes human influenza, is very similar to the experience of the writer in the early filtration experiments with swine influenza. The preliminary obstacles encountered in studying the nature of the etiological factors in swine influenza have had much in common with those met by investigators of human influenza. A careful investigation would seem warranted of the possibility that Pfeiffer's bacillus and a filtrable agent act in concert to cause influenza in man.

SUMMARY AND CONCLUSIONS

1. It has been possible to demonstrate, in Berkefeld filtrates of infectious material from experimental cases of swine influenza, a virus which when administered intranasally to susceptible swine induced a mild, usually afebrile illness of short duration. The changes in the respiratory tract resembled those in swine influenza but were usually much less extensive. When the filtrable virus was mixed with pure cultures of *H. influenzae suis* and administered to swine a disease identical clinically and pathologically with swine influenza was induced. The data presented indicate that the filtrable virus of swine influenza and *H. influenzae suis* act in concert to produce swine influenza and that neither alone is capable of inducing the disease.

2. One attack of swine influenza usually renders an animal immune to reinfection. Blood serum from an animal made immune in this way neutralizes infectious material from swine influenza *in vitro*, as shown by the failure of the mixture to produce disease in a susceptible animal.

3. The virus can be stored in a dried state or in glycerol for several weeks at least. In one instance dried material apparently retained both the virus and *H. influenzae suis* in viable form for a period of 54 days.

4. Fatal cases of experimental swine influenza have been observed

in which *H. influenzae suis* was the only organism that could be cultivated from the respiratory tract.

5. Attention has been called to some features of marked similarity between epizootic swine influenza and epidemic influenza in man.

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THE SPECIFIC CYTOTOXIC ACTION OF TUBERCULIN IN TISSUE CULTURE

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By tissue culture methods Fischer (1) studied the effects of tuberculin prepared from an avian type of tubercle bacillus upon cultures of fibroblasts from the pericardium of tuberculous and of non-tuberculous chickens. He found that fibroblasts from the tuberculous chickens grew more extensively when tuberculin was added than did cultures from the non-tuberculous chickens. Rich and Lewis on the contrary (2) found that the addition of tuberculin from a human type of tubercle bacillus to explants of the spleen or to leucocytes of tuberculous guinea pigs inhibited the growth and migration of the cells but had no effect on the migration and growth of cells from tissues of non-tuberculous animals.

The following experiments were undertaken to ascertain whether tissue culture methods are applicable in determining the specificity of the cytotoxic action of tuberculin prepared from the human, bovine and avian types of tubercle bacilli and from various other strains of acid-fast bacteria.

Method

Human, bovine and avian tubercle bacilli and other acid-fast bacteria were grown upon beef infusion broth containing 3 per cent of glycerol. When the growth was so luxuriant that it fell to the bottom of the flask the culture was heated in an Arnold sterilizer for 2 hours and evaporated over a steam bath to one-tenth of its original volume. The bacillary bodies were removed by centrifugation and the supernatant fluid filtered through a Seitz filter.

The tissue to be cultured was removed under sterile conditions and placed in Locke solution. Plasma was obtained by bleeding guinea pigs or chickens from the heart and mixing the blood with a 0.1 per cent solution of heparin in Locke solution. The blood was centrifugalized in chilled tubes and the plasma transferred to other tubes, which were then packed in ice until ready for use. The embryonic extract was obtained by centrifugalizing finely minced chick embryos from 8 to 10 days and diluting the supernatant fluid with an equal amount of Locke solution.

The action of the different tuberculins was determined by adding varying dilutions of each tuberculin to sterile cover-slips on which minute bits of tissue previously washed in several changes of Locke solution had been placed. A drop of plasma and a drop of diluted embryonic extract were added, mixed with a glass rod and the cover-slip then inverted over the well of a hollow ground glass slide. The edges of the cover-slips were sealed with paraffin and the cultures were incubated at 37°C. As a control explants of the tissues were prepared without tuberculin.

Effect of Old Tuberculin on Guinea Pig Tissues

Explants of bone marrow of either tuberculous or non-tuberculous guinea pigs to which no tuberculin has been added, when examined after 3 to 4 hours' incubation show numerous migrating cells. After 18 to 24 hours' incubation the growth of cells has proceeded for some distance beyond the explant. These cells are large, irregular and actively motile, with fine hair-like or lobose pseudopodia. The protoplasm contains numerous granules both coarse and fine, the latter showing active Brownian motion. When dilutions of 1:15 to 1:120 of old tuberculin from the human or the bovine type of tubercle bacilli are added to explants of bone marrow from a tuberculous guinea pig it will be noted that after 18 to 24 hours' incubation the cells that have either failed to migrate and multiply or the small number of cells that have migrated are degenerated or dead as evidenced by their rounded, shrunken or vacuolated appearance. When dilutions of tuberculin greater than 1:120 are added cell migration is well marked but numerous degenerated and dead cells may be seen as well as numerous actively motile living cells. The number of living cells increases as the concentration of tuberculin is decreased. Tissues from different tuberculous guinea pigs show quantitative differences in their sensitivity to tuberculin.

When tuberculin from mammalian tubercle bacilli or "tuberculin" from other acid-fast bacteria or when glycerol broth concentrated, like the tuberculin, in dilutions of 1:15 was added to explants of bone marrow from non-tuberculous guinea pigs the growth and migration of the cells were inhibited but when these tuberculins or glycerol broth were added in greater dilutions to explants of bone marrow the growth and migration of the cells were as extensive as in control cultures free of tuberculin.

Table I summarizes the results of a typical experiment with explants

of bone marrow from a guinea pig inoculated 4 weeks previously into the subcutaneous tissue with 0.1 mg. of a human strain of tubercle bacilli. As a control explants of bone marrow from a non-tuberculous guinea pig were used. The bone marrow in both instances was planted in the plasma of a tuberculous guinea pig.

TABLE I
Action of Old Tuberculin on Explants of Bone Marrow

Dilution of tuberculin*	Explants of bone marrow of guinea pigs infected with <i>M. tuberculosis</i> human	Explants of bone marrow of non-tuberculous guinea pigs
1:15	No migration or growth	
1:30	" " " "	Slight migration, cell shrunken and rounded
1:60	" " " "	Marked migration, numerous cells with irregular outline, few round, shrunken, vacuolated cells
1:120	Slight migration, small number of rounded, shrunken, vacuolated cells, occasional cell with irregular outline	Extensive migration, cells with irregular line, few rounded, shrunken cells
1:240	Slight migration, cells shrunken, vacuolated, rounded, few cells with irregular outline	" "
1:480	" "	" "
1:960	Moderate degree of migration, numerous shrunken, rounded cells, numerous cells with irregular outline	" "
1:1920	Migration extensive, few shrunken, rounded cells and numerous cells with irregular outline	" "
No tuberculin	Extensive migration, numerous large cells with irregular outline, occasional rounded, shrunken cell	" "

* Tuberculin prepared from *M. tuberculosis* human, diluted in Locke solution.

The cytotoxic action of tuberculin for explants of spleen and testes was also studied. It was found that tuberculin from human or bovine tubercle bacilli in dilutions of 1:15 to 1:120 inhibited the migration and multiplication of macrophages and other cells in the explants of

of the spleen, and of the fibroblasts in the explants of the testes of tuberculous guinea pigs but that in the explants from non-tuberculous guinea pigs dilutions of tuberculin greater than 1:15 did not affect cellular growth or migration.

It was noted that explants from the tissues of tuberculous guinea pigs planted in plasma from non-tuberculous guinea pigs or in Locke solution were equally sensitive to the cytotoxic action of tuberculin prepared from the human or bovine types of tubercle bacilli. On the other hand explants from tissues of non-tuberculous guinea pigs grown in plasma obtained from tuberculous animals were not sensitized to the action of tuberculin. These results, which are in accord with those of Fischer (1) and of Rich and Lewis (2) indicate that sensitivity to tuberculin is a cellular characteristic and cannot be transferred passively.

Specificity of the Cytotoxic Action of Tuberculin

The cytotoxic action of tuberculin prepared from the mammalian types of tubercle bacilli on tissue from tuberculous animals was so definite that further investigation was undertaken to determine the specificity of the reaction.

"Tuberculins" prepared from various acid-fast bacteria were added to explants of the bone marrow and spleen of guinea pigs previously infected with either the human or the bovine type of tubercle bacillus as well as to explants of the same tissues of non-tuberculous guinea pigs.

In one series of experiments the explants were grown in plasma from tuberculous guinea pigs and chick embryonic extract while in a second series the tissues were planted in Locke solution. Similar results were obtained with either method.

With dilutions of 1:30 to 1:640 the "tuberculins" from the acid-fast bacteria other than the human or the bovine type of tubercle bacillus had slight or no effect on the growth of the explants derived from the tuberculous animals and in dilutions greater than 1:15 none of the tuberculins exerted any inhibitory action on the explants from non-tuberculous animals. The specific effect of the "tuberculins" prepared from the various acid-fast bacteria upon explants of tissue from tuberculous and non-tuberculous guinea pigs is shown in Table II.

The specific nature of the toxic action of tuberculin prepared from the mammalian types of tubercle bacilli for tissues of animals previously infected with these bacilli as shown in Table II are in accord with results obtained by other methods. It was found by Ramond and

TABLE II
The Action of Tuberculin from Various Acid-Fast Bacteria on Explants of Bone Marrow

Tuberculin* prepared from the following <i>Mycobacteria</i>	Explants from bone marrow of guinea pig infected with <i>M. tuberculosis</i> human		Explants from bone marrow of non-tuberculous guinea pig		
	No migration, few shrunken rounded cells	" "	Extensive growth	migration	and
<i>M. tuberculosis</i> (human).....	Extensive migration and growth	" "	" "	" "	" "
<i>M. tuberculosis</i> (bovine).....	" "	" "	" "	" "	" "
<i>M. avium</i>	" "	" "	" "	" "	" "
<i>M. piscium</i>	" "	" "	" "	" "	" "
<i>M. marinum</i>	" "	" "	" "	" "	" "
<i>M. chelonae</i>	" "	" "	" "	" "	" "
<i>M. ranae</i>	" "	" "	" "	" "	" "
<i>M. thamnophicos</i>	" "	" "	" "	" "	" "
<i>M. schlangen</i>	" "	" "	" "	" "	" "
<i>M. leprae</i> (Clegg).....	" "	" "	" "	" "	" "
<i>M. leprae</i> (Duval).....	" "	" "	" "	" "	" "
<i>M. leprae</i> (Kedrowsky).....	" "	" "	" "	" "	" "
<i>M. smegmatis</i>	" "	" "	" "	" "	" "
<i>M. mageritensis</i>	" "	" "	" "	" "	" "
<i>M. butyrlicum</i>	" "	" "	" "	" "	" "
<i>M. berolinensis</i>	" "	" "	" "	" "	" "
<i>M. friburgensis</i> (Korn).....	" "	" "	" "	" "	" "
<i>M. stercois</i>	" "	" "	" "	" "	" "
<i>M. phlei</i>	" "	" "	" "	" "	" "
<i>M. pseudoperlsucht</i>	" "	" "	" "	" "	" "
<i>M. paratuberculosis</i>	" "	" "	" "	" "	" "
<i>M. tuberculosis</i> (Koch).....	" "	" "	" "	" "	" "
Concentrated glycerol broth...	" "	" "	" "	" "	" "

* In dilutions of 1:30 to 1:60.

Ravaut (3) and by Ledoux-Lebard (4) that "tuberculin" from *M. piscium* gives a slight reaction or none when injected into tuberculous guinea pigs, and the same observation was made by Beck (5) with *B. tuberculosis*, by Zupnik (6) with *pseudoperlsucht* bacillus, by Dietrich (7) with *M. chelonae*, *M. ranae* and *blindschleichen* bacillus, by Lange

and Lange (8) with *M. phlei*, by Crawford (9) with *M. avium* and by Aronson (10, 11) with *M. thamnophaeos* and *M. marinum*.

The observations with tissue culture are also in agreement with the results obtained by serological methods. Aronson and Lewis (12) found by means of the complement fixation reaction that the human and bovine tubercle bacilli were similar to each other in antigenic structure but that they differed from the avian tubercle bacillus and from the various acid-fast bacteria. In a more precise manner, Wilson (13), and Furth (14) demonstrated by means of agglutination and agglutination-absorption experiments that the antigenic structure of the human and of the bovine tubercle bacilli are identical, and unlike that of the avian and of the other acid-fast bacteria.

Relation of Age of Animal to Sensitivity to Tuberculin

Freund (15) has shown that although young guinea pigs previously infected with the mammalian type of tubercle bacilli show slight if any reaction to tuberculin injected into the skin, yet they are as sensitive as adult tuberculous guinea pigs to the lethal action of tuberculin injected into the peritoneal cavity. This observation suggests that tissues of young guinea pigs are sensitized to the toxic action of tuberculin but that they do not react cutaneously because, presumably, of certain anatomical and physiological differences in the skin of young animals.

To determine whether the sensitivity of the various tissues of the young tuberculous guinea pig to tuberculin differs from that of the adult guinea pigs, two adults and their litters of four 1 day old guinea pigs were injected subcutaneously with 0.1 mg. of a strain of tubercle bacillus of human type. These animals were killed 28 days after infection and explants of the spleen and bone marrow were prepared simultaneously. The tissues were planted in plasma from an adult tuberculous guinea pig and tuberculin from the human type of tubercle bacillus in dilutions 1:15 to 1:240 was added. It was found that the explants from the young and from the adult animals were equally sensitive to the same dilution of tuberculin.

Effect of Old Tuberculin on Chicken Tissues

To observe further the specificity of the different tuberculins studies were made of their action upon explants of spleen and bone marrow of tuberculous and of non-tuberculous chickens. The technique employed was the same as that described in the first part of this paper

except that plasma from tuberculous and non-tuberculous chickens was substituted for plasma from guinea pigs.

The tuberculous chickens used in these experiments were from a flock of naturally infected fowls. They gave a well marked inflammatory reaction when 0.01 cc. of avian, human or bovine tuberculin was injected into the wattle, while the non-tuberculous fowls did not react to this test.

In cultures of the spleen of the tuberculous and non-tuberculous chickens to which no tuberculin had been added, numerous macrophages, lymphocytes and other cells were noted after incubation for 18 to 24 hours. After 48 hours the number of macrophages increased and a number of fibroblasts were seen. In explants of bone marrow with no tuberculin, numerous granulocytes, myelocytes and a small number of macrophages were observed after 24 hours' incubation. Migration and multiplication of cells was more extensive in explants of the spleen than in explants of bone marrow, unlike the guinea pig tissue cultures in which these activities were more marked in the bone marrow.

Tuberculin from the various acid-fast bacteria was added in dilutions from 1:15 to 1:1200, to explants of spleen and bone marrow from tuberculous and non-tuberculous chickens. It was observed after 24 hours that those cultures from tuberculous chickens receiving tuberculin prepared from the avian, the human and the bovine type of tubercle bacillus, from *M. leprae* Duval and *M. leprae* Kedrowsky, in dilutions of 1:15 to 1:60 showed no growth and no migration of the cells. These explants were as sensitive to tuberculin from the mammalian tubercle bacilli and from the two *leprae* strains as to tuberculin prepared from the avian type of microorganism. With dilutions of these tuberculins greater than 1:60 numerous living as well as dead and degenerated cells were observed. Explants of spleen and bone marrow from non-tuberculous chickens were not affected by the addition of these tuberculins in dilutions greater than 1:15. Explants from tuberculous chickens planted in the plasma from non-tuberculous chickens were equally sensitive to the toxic action of tuberculin from those acid-fast microorganisms mentioned above. Explants from non-tuberculous chickens grown in plasma from tuberculous fowls were not sensitized to the action of tuberculin.

Table III shows the effect of different tuberculins upon explants of the spleen of tuberculous and of non-tuberculous chickens.

It will be noted that "tuberculin" prepared from the *M. leprae* Clegg has no cytotoxic effect on explants from tuberculous chickens, in

TABLE III
The Action of Tuberculin from Various Acid-Fast Bacteria on Explants of Spleen of Chickens

Tuberculin* prepared from the following <i>Mycobacteria</i>	Explants of spleen of tuberculous chicken	Explants of spleen of non-tuberculous chicken
<i>M. tuberculosis</i> (human).....	Slight migration of cells	Extensive migration and multiplication
<i>M. tuberculosis</i> (bovine).....	" " " "	" "
<i>M. avium</i>	" " " "	" "
<i>M. piscium</i>	Moderate migration and growth of cells	" "
<i>M. marinum</i>	Extensive migration and growth of cells	" "
<i>M. chelonci</i>	" "	" "
<i>M. ranarum</i>	" "	" "
<i>M. thamnophis</i>	" "	" "
<i>M. schlangen</i>	" "	" "
<i>M. leprae</i> (Clegg).....	" "	" "
<i>M. leprae</i> (Duval).....	No migration or growth of cells	" "
<i>M. leprae</i> (Kedrowsky).....	" "	" "
<i>M. smegmatis</i>	Extensive migration and growth of cells	" "
<i>M. mageritense</i>	" "	" "
<i>M. butyrlicum</i>	Moderate degree of migration	Moderate degree of migration
<i>M. friburgensis</i> (Korn).....	Extensive migration	Extensive migration
<i>M. stercoarius</i>	" "	" "
<i>M. phlei</i>	" "	" "
<i>M. pseudoperlsucht</i>	" "	" "
<i>M. paratuberculosis</i>	" "	" "
<i>M. tuberculosis</i> (Koch).....	" "	" "
Concentrated glycerol broth..	" "	" "

* In dilutions of 1:30 to 1:60.

contrast to the action of "tuberculin" from the *M. leprae* Duval and from the *M. leprae* Kedrowsky. This difference is significant since it furnishes additional proof of the relationship between the Duval

and the Kedrowsky strains of *M. leprae* and the avian type of tubercle bacillus.

In a previous study Lewis and Aronson (16) found by means of complement fixation that bacillary emulsions of avian tubercle bacilli of the Duval and the Kedrowsky strains of *M. leprae* have a similar antigenic character which differs from that of the Clegg strain of *M. leprae* and the acid-fast saprophytes. Furth (14) by means of agglutination and agglutination-absorption experiments concluded that the Duval and the Kedrowsky strains of *M. leprae* are antigenically identical with his third group of avian tubercle bacilli.

DISCUSSION

Tissue culture methods have been used to demonstrate the specific toxic action of tuberculin for tuberculous tissue and have given results in agreement with those obtained by other investigators using either serological methods, or the tuberculin reaction on tuberculous animals. The majority of investigators using serological methods have found that the human and the bovine type of tubercle bacilli have the same antigenic structure. However, Seibert (17) by means of the precipitin reaction has noted differences in the antigenic character of the tuberculo-protein of the two types of tubercle bacilli. The avian tubercle bacilli, *M. leprae* Duval and *M. leprae* Kedrowsky, have been shown to possess a similar antigenic character, which differs from that of the mammalian tubercle bacilli, from the *M. leprae* Clegg, from the tubercle bacilli of the cold blooded animals and from the acid-fast saprophytes.

It has been observed that animals infected with either the human or the bovine type of tubercle bacilli react quantitatively to the same degree of tuberculin prepared from either of the two mammalian types of tubercle bacillus, but do not react to tuberculin prepared from the avian type, from tubercle bacilli of the cold-blooded animals, from various strains of *M. leprae* or from the acid-fast saprophytes. Whereas animals infected with the avian type react not only to tuberculin prepared from this type but react to a lesser degree to tuberculin prepared from the bovine or human type of tubercle bacillus. Crawford (9) has observed that guinea pigs infected with the avian tubercle bacillus react not only to tuberculin prepared from this microorganism but

also react to a lesser degree to tuberculin prepared from mammalian tubercle bacilli. Similar observations have been made by Elder and Lee (18), Plum (19), Mitchell and Duthie (20) on calves and cattle infected with the avian type of tubercle bacillus. Schalk (21) on the other hand found that 75 per cent of cattle exposed to tuberculous fowls became sensitive to tuberculin from the avian tubercle bacillus but did not react to tuberculin prepared from the bovine tubercle bacillus.

The writer has noted that tuberculous fowls give a marked inflammatory reaction and edema when tuberculin from the avian, human, bovine, *M. leprae* Duval or *M. leprae* Kedrowsky are injected into the wattle but that the reaction to the tuberculin from the avian type and from the two strains of *M. leprae* is more intense than to the tuberculin from the mammalian types. Dr. E. L. Stubbs of the School of Veterinary Medicine of the University of Pennsylvania, has observed that tuberculous fowls are more readily desensitized to tuberculin from the mammalian types than to tuberculin from the avian type.

CONCLUSION

Tuberculin from the human and from the bovine type of tubercle bacilli inhibits the growth of cells from explants of bone marrow, spleen and testes of tuberculous guinea pigs, and is toxic for these cells, but has no effect on explants of the same tissues from non-tuberculous animals.

"Tuberculin" from other acid-fast bacteria has no inhibitory or toxic action on explants of tissues from either tuberculous or non-tuberculous guinea pigs.

Tuberculins from the avian, bovine and human types of tubercle bacillus as well as "tuberculin" prepared from the Duval and from the Kedrowsky strains of *M. leprae* inhibit the growth of the cells of explants of the spleen and bone marrow of tuberculous fowls and are toxic for these cells, but have no effect on the explants from tissues of non-tuberculous chickens.

"Tuberculins" from other acid-fast bacteria have no effect on the growth of explants of tissues from tuberculous or from non-tuberculous fowls.

Tissue culture methods indicate that the sensitivity of tuberculous tissues to tuberculin is inherent in the cell, and that it cannot be passively transferred.

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THE HISTOPATHOLOGY OF NUTRITIONAL ENCEPHALOMALACIA OF CHICKS

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PLATES 36 TO 39

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In a recent paper, Pappenheimer and Goettsch (1) described a nutritional disorder of growing chicks, characterized by striking alterations in the brain. While the general features of the lesions were pointed out in this article, further experiments have afforded abundant material for a more detailed and complete histopathological study. The application of neurohistologic methods has brought out features not disclosed by the routine technique, and has thrown light upon the pathogenesis of the lesions and upon the course of their development.

Technique

In addition to such routine stains as hematoxylin-eosin, phosphotungstic acid hematoxylin, Masson's trichrome and Scharlach R for fat, the following neuropathological methods have been employed:—Spielmeyer's method for myelin sheaths, Rio del Horta silver carbonate method for microglia and oligodendroglia, Cajal's gold sublimate method for astrocytes, Bielschowsky's method for axon cylinders, and Laidlaw's reticulum stain.

Histopathology of the Brain Lesions

The salient features originally noted may be summarized as (1) edema, with separation and disruption of the cellular and fibrillar elements; (2) degeneration and necrosis of the Purkinje cells, and of the small cells constituting the granular layer of the cerebellum; (3) small hemorrhages scattered through the cortical white matter, or within the cortical zones; (4) hyaline capillary thrombi in and about the necrotic areas. Emphasis was placed in the previous paper upon the cerebellar lesions, although reference was made to two chicks in which there

occurred lesions of essentially the same character in the cerebrum. The material which has accumulated has taught us that cerebral localization of the lesions is of not infrequent occurrence, and that similar changes may be also found at times in the medulla and midbrain. The relative frequency with which the lesions have been found in the different portions of the brain is shown in Table I.

Since the brains were not serially sectioned, these figures cannot be regarded as very accurate, but they indicate correctly the relative sensibility of the different portions of the brain to the disease, and the predominance of the cerebellar lesions. Lesions of the cerebrum, unaccompanied by cerebellar lesions were found in but thirteen chicks, and isolated lesions of the medulla or midbrain in but three. Only once were lesions found in the optic lobes.

TABLE I

		<i>per cent</i>
Total No. of chicks.....	169	
Cerebellar lesions.....	153	90.6
Cerebral lesions.....	48	28.4
Medullary or midbrain lesions.....	19	11.3

The earliest and mildest recognizable lesions point very definitely to a circulatory disturbance as the initial factor in the production of the lesions. Thus, in the cerebellum, there is seen engorgement of the pial vessels, and of the capillaries of the molecular and granular layers, and of the central white matter. Not all the vessels are filled with erythrocytes; some are dilated or empty. This is seen especially in the white matter, less often in the granular layer, and only occasionally in the lower portion of the molecular layer. Numerous small hemorrhages occur into the pia and the cerebellar layers, but are most conspicuously seen in the lower portion of the molecular layer. A mild edema separates the fibers of the white matter.

A further step in the development of the lesions is seen in the presence of definite edema in the Purkinje cell layer, which becomes strikingly rarefied and spongy (Fig. 6). The Purkinje cells and the Bergmann astrocytes are spread apart, and often displaced away from the granular layer towards the pia. In Bielschowsky preparations, the ascending fibers of the Golgi cells, the basket fibers, and the collaterals of the Purkinje axones which form the plexus infra-ganglionaris, are seen to be spread apart. In Cajal gold sublimate preparations, the large astrocytes of the Bergmann type, instead of forming two or three regular layers at the Purkinje cell level and sending out parallel rows of fibers at right angles to the pia to form

the external glial membrane, are found irregularly arranged and separated by the edema.

The Purkinje cells, Golgi cells, and the small cells of the granular layer are seen undergoing degeneration of the type described by Spielmeyer as "ischemic necrosis" (Fig. 7). A similar change is later seen in the basket cells.

The Purkinje cells lose their Nissl substance, become angular and narrow, and their nuclei pycnotic. Intracellular fibrillae disappear. The other large ganglion cells react similarly. The nuclei of the Bergmann cells are swollen and hydropic (Fig. 8).

The small granular cells show marked pycnosis and shrinkage of their nuclei followed in later stages by karyorrhexis into small spherical fragments. The internuclear protoplasmatic islands stain very faintly. These changes are patchy. A few affected Purkinje cells may be seen in an edematous area, with the adjacent granular layer showing a small group of degenerating cells, or these lesions may be found separately. The extent of the change in the granular layer may vary from a small focus to an area involving the entire granular zone.

At this point, small collections of rod cells appear, chiefly in the molecular layer, but also occasionally in the white matter. Stained by Hortega's silver carbonate method, these are seen to be groups of microglia cells. As compared with the normal, their cell body is thickened and elongated, the nucleus lengthened, and the processes short, reduced in number, distorted, clubbed, and without spines (Fig. 1). The increase in the normal number of these elements is first observed in the lower half of the molecular layer, and later diffusely through this entire zone (Fig. 9). The microglia cells arrange themselves as on a trellis along the outlines of the dendrites of the Purkinje cells (Spielmeyer's "*Strauchwerk*"), probably functioning as neuronophages.

At this, and at earlier stages, the oligodendroglia, as stained by a modification of Hortega's silver carbonate method, lose their processes, but do not undergo the mucoid degeneration which has been described in early degenerative lesions of the mammalian brain (Figs. 2 *a* and 2 *b*).

The alterations of the fibrillar astrocytes are not particularly striking. There is an apparent increase in the number of the Bergmann cells, and probably of their fibers. With the disappearance of the Purkinje cells, the Bergmann fibers, as shown in the Cajal gold sublimate preparations, become concentrated. The pial surface may become sunken over such an area. In the healing stages, the fibers may become incrustated with calcium (Fig. 10).

In the central white matter, especially in the vicinity of the fourth ventricle, one occasionally observes multiplication of the fibrous astrocytes with the formation of many new glia fibers (Fig. 11). In such areas, the myelin sheaths are shown in Spielmeyer-stained sections, to have undergone destruction, and the Bielschowsky preparations bring out the fact that the neuraxons have also disappeared (Fig. 3).

At this stage, the ganglion cells have been almost entirely destroyed, only nu-

clear, protoplasmatic, and fibrillar fragments remaining to indicate their original position. Lipoid droplets are found within these degenerating remnants.

Interesting alterations are seen in the capillaries. The endothelial cells become greatly swollen, and grow actively, often with lateral sprouting, into the necrotic tissue. Mitotic figures are frequently encountered.

From this point on, the astrocytes and oligodendroglia degenerate and disappear (Fig. 4). The tissue becomes more and more spongy, and contains numerous compound granule cells, which in the Scharlach R stain are seen to be laden with fat (Fig. 12). The hyaline thrombi, as described in the previous paper, are present in and about the lesions from the very first appearance of the degenerative changes in the ganglion cells (Fig. 13).

As a final stage in the process, one finds that the malacic areas become more or less organized by the ingrowth of mesodermal tissue. Reticular fibers are seen in increasing abundance in and about the walls of the cortical blood vessels, often constituting compact, tubercle-like nodules. Small focal areas may be organized in this way, or one or several entire lobules. From these, fibers pass out and interlace through the area to form a loose spongy network (Fig. 5). In this are found varying numbers of phagocytes, containing brownish yellow pigment, some of which gives an iron reaction.

It is not to be assumed that the lesions throughout the cerebellum are of necessity all at the same stage of evolution. On the contrary, it is not unusual to find early acute degenerative changes in some of the lobules, while in other lobules or areas, various stages of repair are in progress. One may assume from this fact that the brain has been subjected to repeated or continued insult. On the other hand, in many of the chicks that were killed soon after the onset of symptoms, only the early lesions are discoverable; and if one may draw deductions from the clinical behavior, most extreme changes may develop in a very short period—certainly within 6 hours or less.

The study of the lesions in the cerebrum and medulla adds nothing of importance to what has already been described. Both the degenerative and the reparative processes are essentially identical with those occurring in the cerebellum.

In those cases in which both cerebrum and cerebellum are affected, it has repeatedly been noted that the cerebral lesions are recent in their appearance, while the cerebellar lesions are in the healing or reparative stage. Clearly, the injury to the cerebellum in these cases antedates that in the cerebrum.

DISCUSSION

It was stated in the previous paper that the lesions in the chicks were probably initiated by vascular disturbances, and further morphological study tends to confirm this view. Summarizing the changes described above, we find a degenerative lesion, characterized by edema, rapid necrosis of the neural elements, and later of the astrocytes and oligodendroglia. As reactive changes, one may list the multiplication and local increase of the microglia, with subsequent transformation into compound granular cells, the proliferation of endothelium with the formation of new vascular ingrowths into the degenerated areas, and finally, the partial mesodermal organization of the softened tissue. This series of changes is characteristic of the encephalomalacic areas which are produced in the central nervous system by functional or anatomic vascular disease.

In the disease of chicks, changes in the larger vessels may be excluded as a possible cause of the lesions. While hyaline and fibrin thrombi are almost invariably to be found within and about the degenerated areas, not all the capillaries are thus occluded and we have not been able to bring proof that the capillary thrombosis is a primary cause of the ensuing necrosis. Indeed, it may well follow upon a prolonged vasoconstriction, or vasomotor paralysis, or the first followed by the second.

As an indication of the functional nature of the disturbance, we have found marked symptoms without detectable histological lesions in thirteen chicks. There have been noted tremors, ataxia, retropulsive movements, and in a few chicks, retraction of the head. In the absence of complete serial sections, one cannot be entirely certain that small lesions may not have been present.

Spielmeyer (2) has shown that exactly similar lesions to those here described may occur, not only as a result of gross vascular disease, but in cases in which no anatomic changes are demonstrable and in which one is forced to assume a functional circulatory disturbance. Ricker (3) has long held the view that various factors, mechanical or chemical, may affect the vasomotor nerves, producing vasoconstriction and dilatation of sufficient duration to bring about local necrosis of tissue.

In his study of epilepsy, Spielmeyer (4) has come to the conclusion

that the local lesions often found in that condition, are the result of functional vascular disturbance. He cites the observations of Horsley, Hartwell and Kennedy, Foerster, and others who have actually seen constriction of the cerebral vessels during epileptic seizures. He points out the similarity between the lesions in the Sommers sector of the hippocampus found in epilepsy, unassociated with alterations of the vessel walls, and those which result from gross disease of the vessels supplying that area. It is particularly interesting that Spielmeyer's description of the early cerebellar lesions occurring in epilepsy, applies in all respects to the lesions which we have noted in the chicks.

As an explanation of the greater susceptibility of the cerebellum to circulatory injury, Spielmeyer calls attention to the rather sharp right angled turn of the vessels at the Purkinje cell level, as described by Pfeifer (5). In the cerebellar lesions of the chicks, the early changes are most frequently detected in and about the Purkinje layer, and it seems not unreasonable to believe that this preferential localization may be explained in this way.

CONCLUSION

Whatever may be the nature of the nutritive error or deficiency which in chicks is responsible for encephalomalacia, the immediate cause must be looked for in some agent or condition which impairs the capillary circulation of the brain. The essential lesion is an ischemic necrosis, followed, if the animal survive, by reparative organization of the dead tissue.

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EXPLANATION OF PLATES

PLATE 36

FIG. 1. Increase in number of microglia cells, with elongation of bodies and nuclei and loss and distortion of cell processes. Del Rio Hortega's silver carbonate stain for microglia. Obj. 16 mm. Oc. 10.

FIG. 2 *a*. Section through cerebellar cortex showing normal oligodendroglia.

FIG. 2 *b*. Similar section from encephalomalacic chicks, showing changes in oligodendroglia. Loss of cell processes. Del Rio Hortega's silver carbonate stain for oligodendroglia. Obj. 16 mm. Oc. 10.

FIG. 3. Degeneration of neuraxones in white matter. Bielschowsky stain. Obj. 4 mm. Oc. 10.

PLATE 37

FIG. 4. Destruction of astrocytes in degenerated area without any reaction. Cajal gold-sublimate stain. Obj. 4 mm. Oc. 10.

FIG. 5. Connective tissue replacement of degenerated lobule with formation of nodular areas of condensation in cortex. Laidlaw stain. Obj. 4 mm. Oc. 10.

PLATE 38

FIG. 6. Edema and rarefaction of Purkinje cell layer. Degeneration of cells of granular layer. Perivascular hemorrhages. Hematoxylin and eosin stain. $\times 138$.

FIG. 7. "Ischemic" necrosis of Purkinje cells. Pycnosis of nuclei of granular layer. Edema. Hematoxylin and eosin stain. $\times 618$.

FIG. 8. Swollen and hydropic nuclei of Bergmann cells. Hematoxylin and eosin stain. $\times 618$.

FIG. 9. Marked increase in microglia cells in molecular layer. Hematoxylin and eosin stain. $\times 138$.

PLATE 39

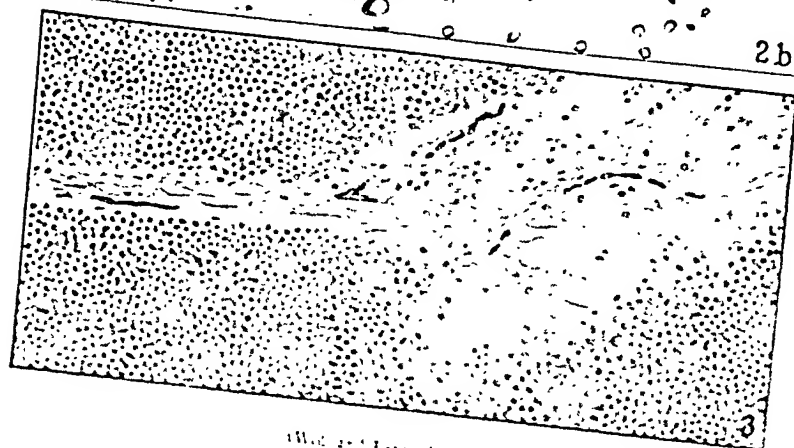
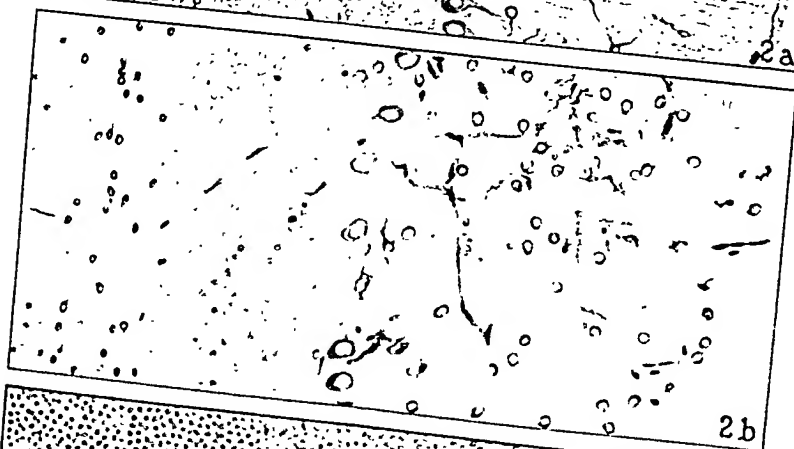
FIG. 10. Incrustation of Bergmann fibers with calcium. Hematoxylin and eosin stain. $\times 618$.

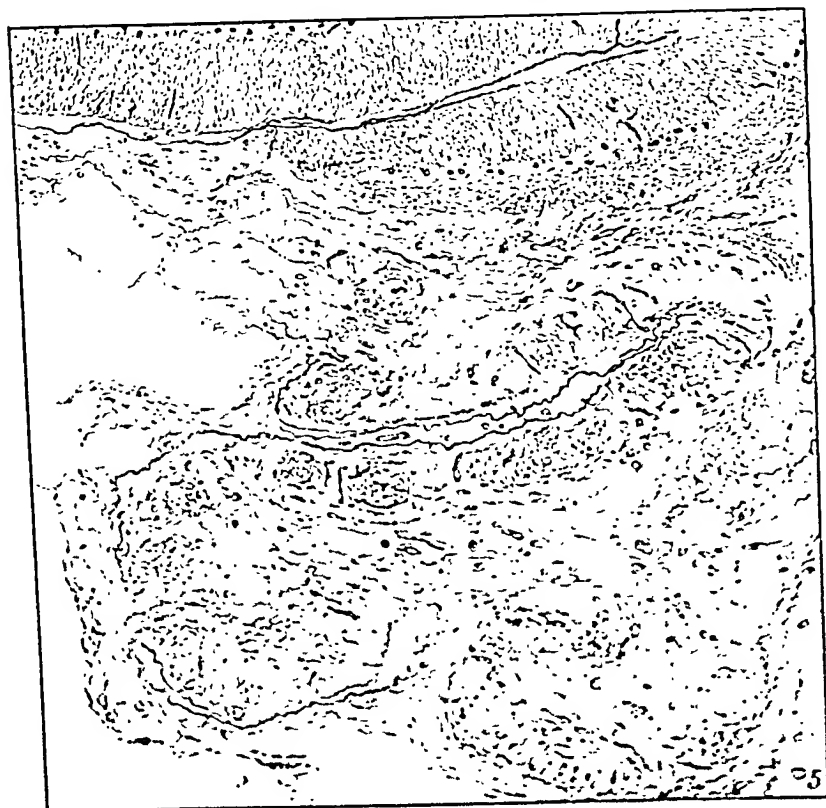
FIG. 11. Increase of astrocytes in white matter near ventricle. Note also destruction of granular and accumulation of microglia in molecular layer. Hematoxylin and eosin stain. Low power. $\times 138$.

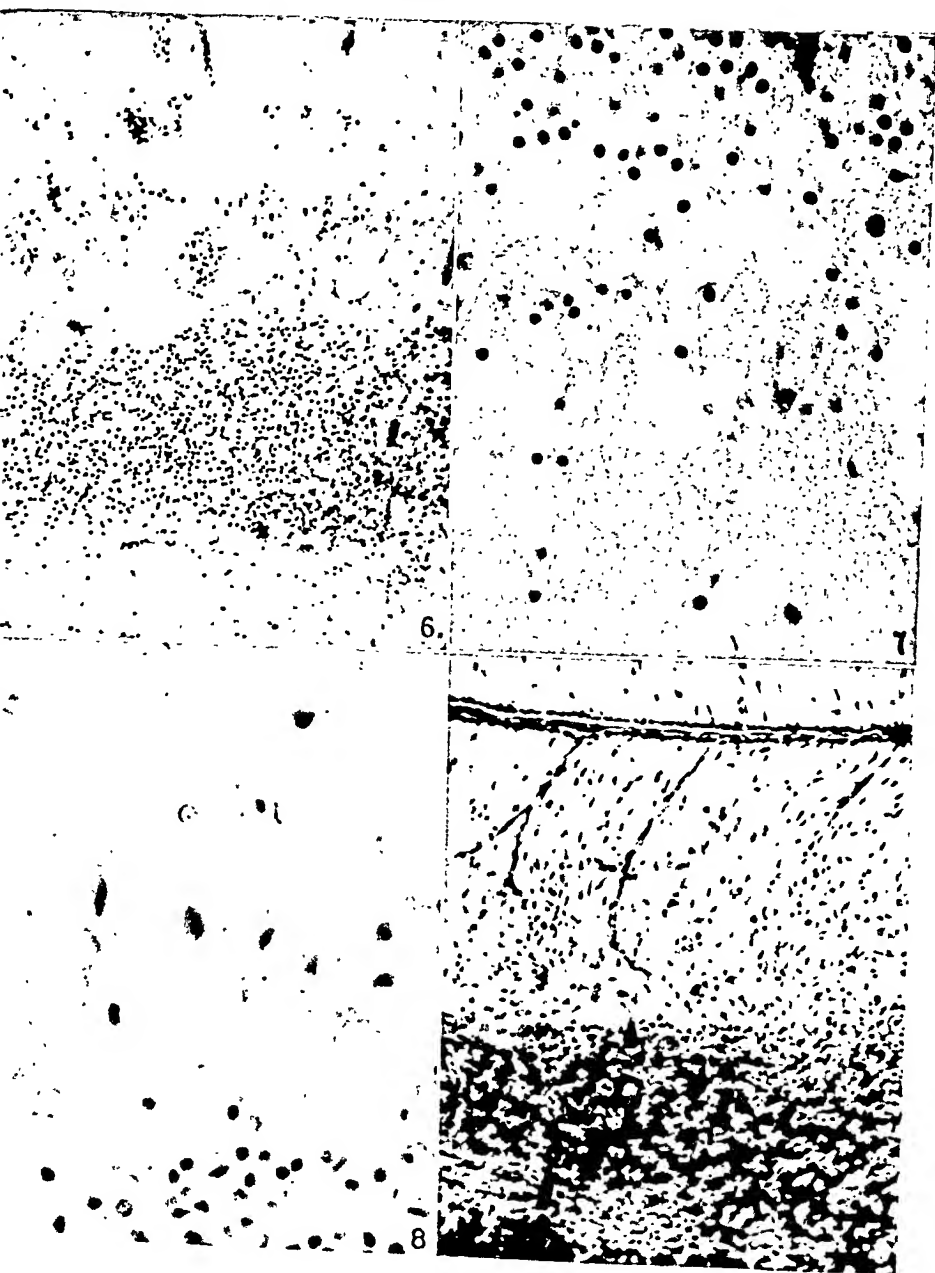
FIG. 12. Atrophy of lobule with appearance of large numbers of lipoid-containing phagocytes. Hematoxylin and eosin stain. $\times 138$.

FIG. 13. Hyaline thrombi in capillaries in degenerated area. Hematoxylin and eosin stain. $\times 138$.

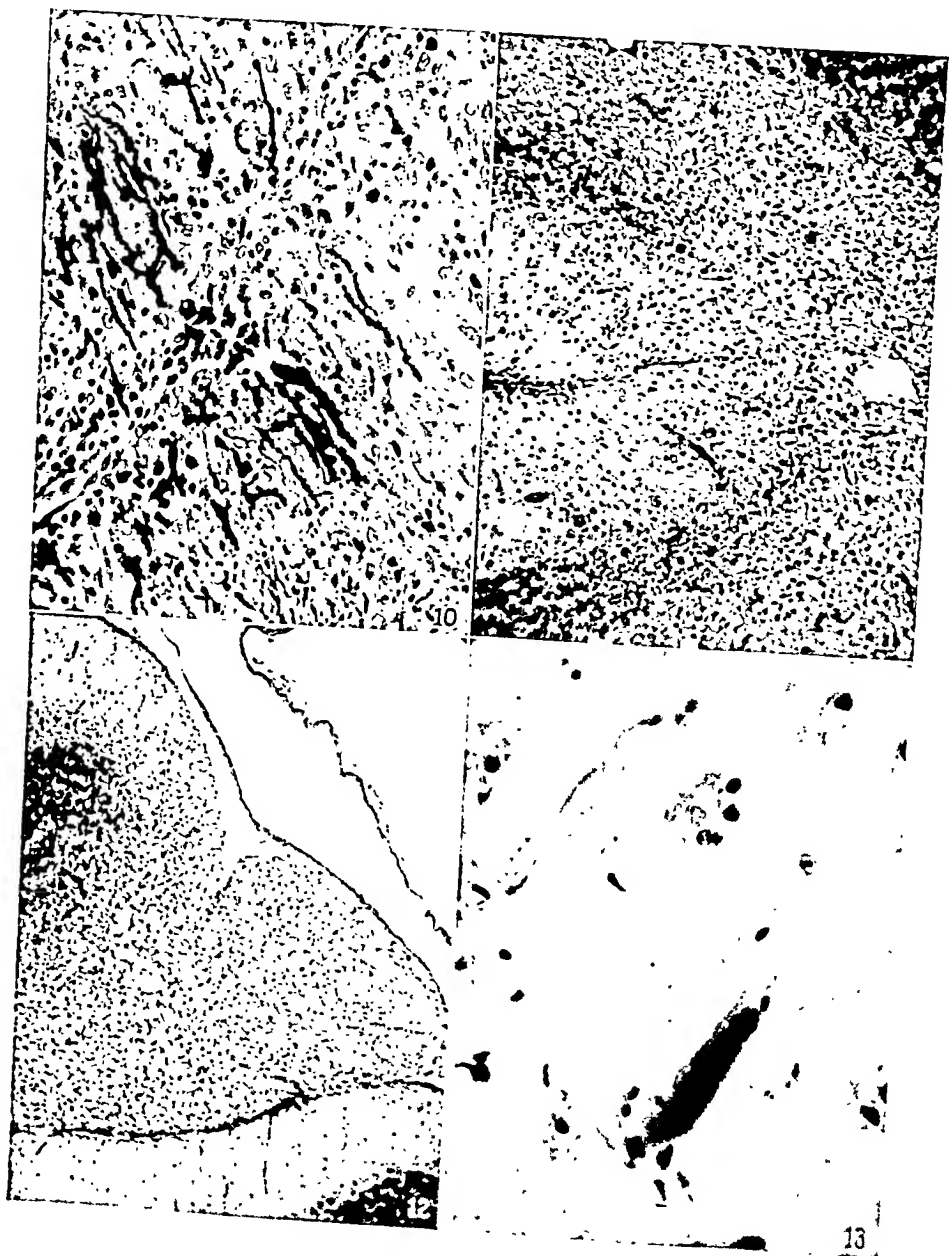
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PRODUCTION OF A REFRACTORY STATE AS CONCERNS THE SHWARTZMAN PHENOMENON BY THE IN- JECTIONS OF VENOM OF THE MOCCASIN SNAKE (ANCISTRODON PISCIVORUS)

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In 1928 Schwartzman (1) described a phenomenon of local skin reactivity to bacterial filtrates which was produced when intradermal injections of these filtrates were followed at a suitable interval by intravenous injections. This observation has been confirmed by several observers (2). Very early in his work Schwartzman noticed that all normal rabbits did not yield the phenomenon. The number of negative animals within limits depended on the amount, concentration and the type of "agar washings" filtrates used. Therefore, in titrating these "agar washings" filtrates and antisera by his method it was necessary to use groups of from three to five rabbits, in order to correct any error due to refractory animals.

We conceived the idea of testing the reactivity of the rabbit skin to substances producing primary purpura to see if a relationship could be established between the intensity of the purpura produced and the susceptibility of the animals to the Schwartzman phenomenon.

Our experiments seem to show that animals developing an intense purpura following intradermal injections of snake venom (*Ancistrodon piscivorus*) in dilutions of 1:1000 and 1:2000 were more likely to develop a Schwartzman phenomenon than those giving a slight reaction.¹ In the course of this work it was observed that if a period of 14 days to 1 month was allowed to elapse between the time of grading with snake venom and the production of a Schwartzman phenomenon a large number of the rabbits became refractory. The following experiments were carried out in order to study this point more fully.

¹ This will be reported in a separate publication.

Method

Method of Grading.—The venom was supplied to us in solid scales by the Antivenin Institute of America. It was used in dilutions of 1:1000 and 1:2000 in physiologic salt solution. The skin on the abdomen of the rabbits was epilated with barium sulfide and 24 hours were allowed to elapse before grading was attempted.

The epilated area at the extreme upper right corner (usually a few inches below the costal margin) received an intradermal injection of 0.1 cc. of 1:1000 snake venom. The reaction was read in 1 to 2 hours. The snake venom produced a reaction in the rabbits which varied from a slight redness to an intense purpura several centimeters in diameter in the more susceptible animals. In the center of the purpuric area there was very often seen a yellowish spot. The yellow area usually indicated an early necrosis. The animals were graded from 0 to 25 according to the degree of reaction which was produced. A slight erythema was 5; redness was graded from 10 to 15 depending on its intensity, and a definite purpura was 20. When the area of purpura was very large or showed a central yellowish spot it was given the maximum grading of 25. Animals giving a zero reaction were rare.

Two venoms were used—those of *Ancistrodon piscivorus* and *Crotalus atrox*; it was found that the venom first mentioned gave the most uniform and reliable results.

Toxins Used.—*B. typhosus*, *B. coli* and meningococcus “agar washings” filtrates were used in the experiments; they were prepared according to Schwartzman’s method (3). *B. typhosus* Filtrates 1229, 1609 and A 53 were used which were prepared from cultures T_L; *B. coli* Filtrate (A 43) prepared from a stock culture of colon bacilli; meningococcus “agar washings” Filtrate 1405 prepared from Wadsworth Strains 44 B (Group III) and 44 D and Filtrate 123 prepared from Washington Hygienic Laboratory Strain (Group I).

In carrying out the test for the Schwartzman phenomenon 0.25 cc. of the undiluted “agar washings” filtrates was injected intradermally in the abdominal skin of a rabbit 24 hours after epilation. 24 hours after the intradermal injection the diluted “agar washings” filtrate — 1 cc. per kilo — was given intravenously. The bacterial filtrates used had been titrated in each instance. According to Schwartzman’s specifications, one unit of “agar washings” filtrate is contained in 1 cc. of the highest dilution which upon intravenous administration in amounts of 1 cc. per kilo, still produces the phenomenon in one or more of three rabbits prepared with 0.25 cc. of the undiluted filtrate. In the tables the number of units given were thus computed.

The site for the Schwartzman phenomenon was always at least 5 cm. removed from the area where the venom was injected and at least 7 cm. separated the various sites for the elicitation of the Schwartzman phenomenon if more than one was done in the same rabbit. In many of the rabbits the area in which it was attempted to elicit the phenomenon was 10 cm. removed from the venom site.

TABLE I
The Refractory State after the Injection of Moccasin Venom

			Shwartzman phenomenon with				
Toxin.....			Typhoid 1229		Meningo- coccus 123 (Group I) 1½ units	Coli A 43	
Dosage per kg.....			1½ units			18 units	
Date.....			Nov. 13-14	18-19	Nov. 21-22	Nov. 25-26	Dec. 5
Rabbit No.	First grading						Regraded 1000 2000
	Date	1000 2000					
663	Nov. 5	15 5	—	0	0	0	5 5
739	Nov. 5	25 20	—	0	0	0	Dead
764	Nov. 5	10 0	—	0	0	0	Dead
771	Nov. 5	15 15	—	0	0	0	15 15
835	Nov. 5	25 25	—	0	0	0	15 10
405	Nov. 7	5 5	—	0	0	0	Dead
960	Nov. 7	20 10	—	0	0	0	20 10
311	Nov. 11	25 20	++++ 1 x ½ cm.	0	0	0	20 5
390	Nov. 11	15 10	+++ 1 x 1 cm.	0	0	0	Dead
445	Nov. 14	20 20	—	0	0	++++ 2½ x 1½ cm.	Dead
Controls							
429			—	—	—	++++ 4 x 3 cm.	Dead
910			—	—	—	+++ 1 x ½ cm.	10 10
970			—	—	—	0	15 10
2			—	—	—	++++ 2 x 2 cm.	25 5

— equals phenomenon not elicited.

*The Effect of Injections of Snake Venom on the Incidence of the
 Shwartzman Phenomenon.*

Series I.—

Experiment 1.—Fourteen rabbits were used in this experiment; ten of the animals were graded with *Ancistrodon piscivorus* venom, according to the technique described above and four were used as controls. After an interval of 2 to 20 days

the attempt was made to elicit the Shwartzman phenomenon with typhoid "agar washings" filtrate (No. 1229) containing 700 units per cubic centimeter, *i.e.* in a dilution of 1:700 1 cc. contained one unit, with meningococcus "agar washings" filtrates titrating 400 units per cubic centimeter and with *B. coli* "agar washings" filtrate (A 43) titrating 1350 units per cubic centimeter.

As can be seen from Table I, no purpura developed in any of the ten rabbits which had received intradermal injections of snake venom 6 to 13 days previous to the attempted elicitation of the Shwartzman phenomenon with one and one-sixth units of the typhoid "agar washing" filtrates intravenously. The same intravenous dose given 2 days after grading gave a positive result in both animals in which it was tried (Nos. 311 and 390). When we attempted to elicit the phenomenon in the same animals 9 to 16 days after grading with the meningococcus filtrates, using one and one-third units per kilo intravenously, only one of the ten animals gave a plus-minus reaction. The experiments in which less than two units are used intravenously per kilo are not very striking because in normal rabbits only about 25 to 30 per cent would give a phenomenon.

13 to 20 days after grading an attempt was made to produce the phenomenon using eighteen units of *coli* "agar washings" filtrates per kilo intravenously and in only one animal did we get a positive reaction. The four controls injected intravenously with the same dosage of *coli* filtrates for the elicitation of the phenomenon gave three positives. When eighteen units of the bacterial filtrates are given intravenously per kilo about 75 per cent positives can be expected in normal rabbits.

A second injection of venom 23 to 30 days after the first gave but a slight variation from the original grading.

Experiment 2.—Ten rabbits were graded with *Ancistrodon piscivorus* venom, while eight rabbits were used as controls. This experiment is essentially the same as Experiment 1 with the exception that the number of units of "agar washings" filtrates given intravenously for the elicitation of the Shwartzman phenomenon was greater.

As can be seen from Table II, when eighteen units of the *B. coli* "agar washings" filtrates were given per kilo intravenously to produce the phenomenon in periods of 14 to 21 days, after grading with snake venom, three of the ten rabbits gave a positive reaction. Of five con-

TABLE II

Influence of Intradermal Injection of Moccasin Venom (*Ancistrodon piscivorus*) on the Incidence of the Stewartman Phenomenon after Large Doses of Toxins

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A nonlethemic after Large Doses of Toxins	Toxin Dose per kg.	Grading	Grading	Coli A 43 18 units	Grading	Typhoid A 53 34 units	Grading	Meningococcus 1405 350 units	
Date	1000	2000	Dec. 5	Dec. 12	Dec. 26-27	Dec. 28	Dec. 30-31	Jan. 6-7	
Injection of ven. in intradermal	1000	2000	1000	2000		1000	2000		
Rabbit No.									
424	15	15	25 25 20 15 20 15 20 20		++++ 2 x 1/2 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
430	15	10	25 25 20 15 20 15 20 20		0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
436	20	10	25 25 20 15 20 15 20 20		++++ 1 1/2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
520	20	20	25 25 20 15 20 15 20 20		0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
526	20	15	25 25 20 15 20 15 20 20		++++ 1/2 x 1/2 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
751	20	20	25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
670			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
802			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
860			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
957			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
Controls			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
657			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
659			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
661			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
683			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
689			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
912			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
916			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	
971			25 25 20 15 20 15 20 20		++++ 3 x 2 cm. ++++ 2 x 1 cm. 0	20 15 10 25 20 15 15 5 25 20 15 10 25 15 Dead	++++ 2 x 1 1/2 cm. 0 0 ++++ 3 x 1 cm. 0 ++++ 2 x 1 cm. 0	0 0 ++++ 3 x 2 cm. ++++ 2 x 2 cm. Dead 0 0 Dead 0	

trols receiving the same dosage, three were positive. Thirty-four units of typhoid bacterial filtrate were given intravenously per kilo 18 to 25 days after grading and four of nine animals were positive; while of five controls four gave a phenomenon.

When 350 units of meningococcus filtrates were given per kilo, 25 to 32 days after injection with snake venom, two of the seven rabbits were positive, while of the eight controls seven were positive.

The first five controls were injected with venom 2 days after the elicitation of a Shwartzman phenomenon with *B. coli* "agar washings" filtrates. When thirty-four units of typhoid filtrates or 350 units of meningococcus filtrates were given intravenously per kilo, a period of 2 to 9 days after the venom injection was not sufficient to permit a resistance to develop in the animals against such large amounts. Animals which became refractory to the phenomenon after an incubation period of about 14 days following the injection of snake venom, tended to remain refractory when the same approximate amount of another bacterial filtrate was used for elicitation of a second Shwartzman phenomenon. On the other hand, those animals which failed to become refractory tended to remain non-refractory even after longer incubation periods.

Experiment 3.—Three rabbits received two intraperitoneal injections of 0.4 cc. of 1:2000 moccasin venom at 8 day intervals; two other rabbits were given similar amounts subcutaneously at the first injection and intramuscularly 8 days later. Five rabbits received two injections of 0.4 cc. of the moccasin venom intravenously a week apart. The attempt was then made to elicit the Shwartzman phenomenon as in Table III.

As can be seen from Table III in two of the intraperitoneally injected rabbits, twenty-five units of meningococcus, typhoid or *coli* "agar washings" filtrates failed to elicit a phenomenon 27, 38 and 45 days after the injections of venom respectively. In one animal twenty-five units of meningococcus filtrate did give a well marked purpura 25 days after the intraperitoneal injection. No conclusion can be drawn from the subcutaneously and intramuscularly injected animals because of the small number of animals used. Only one of the five rabbits which had received moccasin venom intravenously gave a three plus reaction with twenty-five units of meningococcus filtrates per kilo.

Experiment 4.—Each of five rabbits received intradermal injections of snake venom (*Crotalus atrox*) on Jan. 14th and 23rd and Feb. 7th, as in the previous experiments (Experiments 1 and 2). An attempt was made to produce a Schwartzman phenomenon 36, 47 and 54 days after the first intradermal injection of snake venom, with meningococcus, typhoid and *B. coli* "agar washings" filtrates respectively.

TABLE III

Incidence of the Schwartzman Phenomenon after Intraperitoneal, Intramuscular and Intravenous Injection of Venom in Rabbits

		Schwartzman phenomenon with		
Toxin.....	Each injection consisted of 0.4 cc. of 1:2000	Meningococcus 44 D	Typhoid A 53	<i>Coli</i> A 43
Dosage per kg.....	Moccasin venom	25 units	25 units	25 units
Date.....	Jan. 23 and Feb. 7	Feb. 19-20	Mar. 2-3	Mar. 9-10
Rabbit No.	Venom injection route			
610	(1) subcutaneous	+++ $\frac{1}{2} \times \frac{1}{2}$ cm.	0	++++ 2 x 2 cm.
781	(2) intramuscular	0	0	0
826	Intraperitoneal	(0)*	0	0
847		(0)*	0	0
Date.....	Mar. 13 and 20	Apr. 7-8		
107	Intraperitoneal	+++ 2 x 3 cm.	4 other intraperitoneally injected animals died	
41	Intravenous	0		
60		0		
90		0		
97		+++ 2 x 2 cm.		
117		0		

* Animals previously used in meningococcus experiments.

Analysis of Table IV shows that after three intradermal injections of rattlesnake venom twenty-five units of the meningococcus "agar washings" filtrates intravenously per kilo elicited a phenomenon in three of five animals, after a period of 36 days following the first intradermal injection; twenty-five units of the typhoid "agar washings" filtrates elicited a phenomenon in two of four animals after 47 days and twenty-five units of the *B. coli* "agar washings" filtrates gave a

purpura in two of four animals after 54 days. The incidence of positive animals was reduced very little when compared to non-graded rabbits. This experiment seems to show that rattlesnake venom is not effective in producing a refractory state as concerns the Schwartzman phenomenon.

A summary of Series I shows that twenty rabbits received one intradermal injection of *Ancistrodon piscivorus* venom in amounts of 0.1 cc. of the 1:1000 at one site and the same amount of 1:2000 at

TABLE IV

Incidence of the Schwartzman Phenomenon after the Injection of Rattlesnake Venom

Toxins.....	Dosage per kg. 25 units throughout			Schwartzman phenomenon with		
				Meningococcus 44 D	Typhoid A 53	Coli A 43
Date.....	Jan. 14	Jan. 23	Feb. 7	Feb. 19-20	Mar. 2-3	Mar. 9-10
Rabbit No.	Grading with <i>Crotalus atrox</i> 1000 and 2000 respectively					
562	15 5	25 15	15 10	++++ 1 x $\frac{1}{2}$ cm.	++++ 2 $\frac{1}{2}$ x 2 cm.	++++ 2 x 3 cm.
626	15 10	20 20	20 20	+++ 1 x $\frac{1}{2}$ cm.	0	++++ 2 x 3 cm.
655	15 10	20 20	20 15	0	++ 3 x 2 cm.	0
672	20 15	20 15	20 20	++ 3 x 2 cm.	Dead	
681	25 15	20 20	20 20	0	0	0

another. In ten of these rabbits no Schwartzman phenomenon was elicited with a little more than one unit of typhoid or meningococcus "agar washings" filtrates intravenously per kilo, 6 to 9 days after the injection of the snake venom. When eighteen units of *B. coli* bacterial filtrates per kilo were given intravenously in only four out of twenty rabbits did we get a purpura in 13 to 22 days after grading. The same amount of bacterial filtrates intravenously given to nine controls for the elicitation of a phenomenon gave positive results in six out of nine animals. When the dose of the filtrates which was given intravenously was increased—thirty-four units of

typhoid "agar washings" filtrates per kilo—out of nine animals elicited 18 to 25 days after preparation four were positive. With the same technique, of five controls four were positive.

Seven rabbits which were prepared with venom in the usual manner were tested with 350 units of meningococcus "agar washings" filtrates per kilo 25 to 32 days after the intradermal injection with snake venom and only two gave a purpura; seven of eight controls receiving the same amount of bacterial filtrates were positive in this respect.

It can be seen from these experiments that an incubation period of 9 to 14 days is necessary for development of a refractory state as concerns the phenomenon. However it should be noticed that this state was not always complete since, as seen from the protocols (Tables I and II), rabbits failing to react to eighteen units showed a marked purpura when larger amounts of the "agar washings" filtrates were given intravenously.

The efficiency of the intraperitoneal, subcutaneous and intramuscular routes of injection of snake venom for the production of a refractory state should be tested further as the numbers of animals used do not warrant any conclusions. Since out of five animals prepared by the intravenous route only one gave a three plus reaction when twenty-five units of meningococcus "agar washings" filtrates per kilo were given intravenously, it is very probable that this route too is efficacious in producing a refractory state to the Schwartzman phenomenon. The refractory animals did not show any essential change in degree of reaction to a second intradermal injection of snake venom in the concentration used.

Series II.—The experiments about to be described were designed to study the mechanism by which injections of snake venom produce the refractory condition.

Experiment I.—It is conceivable that the injection of moccasin venom produces circulating antibodies which neutralize the Schwartzman intravenous factor. These antibodies may or may not be akin to antivenin. In order to test out this hypothesis five rabbits were injected intradermally as usual with 0.25 cc. of meningococcus "agar washings" filtrate (44 D)—1 cc. containing 1450 units. Two prepared rabbits each received sixty units of the "agar washings" filtrates plus 0.5 cc. of pooled antivenin (Arctic Crotalidae Lot 48804-I supplied by the Antivenin Institute of America) per kilo intravenously while three of the pre-

pared animals received 100 units intravenously plus 0.5 cc. of the antivenin. One of the animals died; three had a four plus reaction and one was a plus-minus.

This experiment shows that antivenin does not neutralize the intravenous factor in the Shwartzman phenomenon and therefore antivenin-like antibodies circulating in the blood stream are not the cause of the refractory state.² It was decided to see whether antivenin would prevent the occurrence of the phenomenon after an incubation period.

Experiment 2.—In order to study this five rabbits each received 2 cc. of pooled antivenin (Nearctic Crotalidae Lot 48804-I) subcutaneously. 2 days later the phenomenon was elicited with twenty-five units of meningococcus "agar washings" filtrates per kilo intravenously. One animal died before the reaction could be read and the other four all developed a four plus reaction.

It seems that antivenin in relatively large quantities even during a period of 48 hours does not produce a passive resistance as concerns the phenomenon.

Experiment 3.—To test the view further that there were no circulating antibodies developed by the rabbits, Rabbits 626, 681, 562 and 672 were bled on Feb. 18. These animals had previously been injected intradermally with rattlesnake venom (Series I, Experiment 4). The serums of 626 plus 681 and 562 plus 672 were pooled. A Shwartzman phenomenon was elicited in six rabbits with meningococcus "agar washings" filtrate (44 D—titration 1:1460). Twenty-five units of the "agar washings" filtrates plus 0.25 cc. of Serum 1 (626+681) were given per kilo intravenously to three animals and Serum 2 (562+672) to the other three in like quantities with the meningococcus toxin. A phenomenon could be elicited in all of the six animals.

No circulating antibodies which would neutralize the Swartzman intravenous factor could be demonstrated by this experiment. However, this may have been due to the fact that the rattlesnake venom was not efficacious in producing the refractory condition. Therefore the following experiment was added:

² To see whether snake venom could raise resistance to true exotoxins, twelve guinea pigs received two intradermal injections of moccasin venom in the skin of the abdomen 0.1 cc. of 1:1000 and 1:2000—on Jan. 28, 1931, and Feb. 7, 1931. On Feb. 27 they each received 2 m. l. d. of diphtheria toxin (1 cc. — 125 m. l. d.). All of the animals died in 2 to 5 days. We could not elicit a Shwartzman phenomenon with diphtheria toxin.

Rabbits 90 and 97 of Experiment 3, Series I, were bled on Apr. 11th. These were the rabbits which had received an intravenous injection of moccasin venom. Rabbit 90 was a resistant animal, Rabbit 97 was not. The Schwartzman phenomenon was elicited in six rabbits with meningococcus "agar washings" filtrate (44 D). Each of three animals received twenty-five units of the bacterial filtrates plus 0.25 cc. of one of the serums per kilo intravenously.

Two of the three rabbits receiving the serum from No. 97 (Schwartzman phenomenon three plus) gave a four plus reaction. All of the three rabbits receiving serum from Animal 90 (refractory animal) gave the phenomenon.

It is conceivable that antivenin injected simultaneously with the skin preparatory factor might have an influence on the elicitation of a Schwartzman phenomenon. To study this the following experiment was made.

Experiment 4.—(a) Pooled antivenin (Nearctic Crotalidae Lot 48805-I) was mixed with equal parts of meningococcus "agar washings" filtrate (M 1044) titrating 1450 units to the cubic centimeter. Each rabbit received 0.5 cc. of the mixture intradermally as usual. 24 hours later each of the animals received twenty-five units of the meningococcus "agar washings" filtrates per kilo intravenously. All of the rabbits yielded negative results.

(b) 10 days later the same experiment was repeated on three of the rabbits but typhoid filtrate (T 1609) was substituted for the meningococcus filtrate. One of the animals was negative while in the other two a phenomenon could be elicited.

(c) The same procedure was repeated on six new animals with antivenin plus *B. coli* filtrate (A 43) titrating 1350 units per cubic centimeter. Four of the animals showed a four plus reaction and two were three plus.

The simultaneous injection of antivenin with typhoid or *coli* bacterial filtrates did not prevent the Schwartzman phenomenon. The inability to elicit a phenomenon in (a) was explained by the presence of meningococcus antibodies in the horse antivenin. This was shown by the demonstration of weak agglutinins against meningococci in the antivenin. Schwartzman had demonstrated in previous experiments that meningococcus antibodies are often to be found in normal horse serum.

COMMENT

Intradermal, intravenous or intraperitoneal injections of moccasin venom produced in the majority of rabbits a refractory state as concerned the experimental purpura known as the Schwartzman phenomenon.

enon, after an incubation period. Evidently the refractory condition, in some of the rabbits at least, is incomplete because it could only be demonstrated in those when smaller doses of the "agar washings" filtrates relatively to the ordinary were given intravenously for the elicitation of the phenomenon.

The Shwartzman phenomenon probably depends on the enhanced vulnerability of the capillaries at the site of the primary injection to a subsequent intravenous injection of the bacterial filtrate. It is conceivable that by injections of small quantities of a vascular poison such as snake venom (6) a change is produced which raises the threshold for the elicitation of the phenomenon.

The refractory condition could not be transferred passively either by means of serum from rabbits made refractory with venom or by antivenin. These facts coupled with the lack of a changed reaction in the refractory animals to snake venom makes it very probable that the mechanism of resistance does not depend on circulating antibodies or on the production of an antivenin.

SUMMARY

The majority of rabbits receiving intradermal, intraperitoneal or intravenous injections of moccasin venom became refractory to the development of the Shwartzman phenomenon. An incubation period of about 14 days was required for their resistance to develop. The incidence of refractory animals was inversely proportional within limits to the amount of toxin given intravenously to elicit the Shwartzman phenomenon. The intravenous route was the most efficacious in developing refractivity. The refractory state was still present 44 days after the primary injection of moccasin venom.

Rattlesnake venom was not efficacious in inducing a refractory state.

The refractory animals did not show a changed reaction to moccasin venom in the concentrations used.

No circulating antibodies could be demonstrated to explain the refractory state.

Antivenin had no effect on the course of the Shwartzman phenomenon.

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IS THE RAT DERMATITIS CONSEQUENT ON VITAMIN B₂ (G) DEFICIENCY TRUE PELLAGRA?*

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PLATES 40 AND 41

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When rats are deprived of water-soluble vitamin B complex they quickly succumb with a variety of pathological manifestations. If the rats are adequately supplied with vitamin B₁ they usually survive much longer but many, sooner or later, develop a type of dermatitis.

The character of this dermatitis led the late Dr. Goldberger and his coworkers (1) to identify it with pellagra and to postulate the existence, in the complex we called water-soluble vitamin B, of a pellagra-preventive substance (P-P). In the years immediately following Dr. Goldberger's suggestion there accumulated evidence of the existence of a new vitamin which the English designate as vitamin B₂ and which in American literature is more commonly designated as vitamin G, also evidence supporting Goldberger's contention that this new vitamin was the pellagra-preventive factor.

At the present writing, however, the view that vitamin B₂ (G) is the significant factor concerned in pellagra prevention is being challenged. Aykroyd (2), Hunt and Wilder (3), Bliss (4), and Sure, Smith, and Kik (5), have recently published papers bearing on this point.

During the past 3 years our laboratory has been especially concerned with the chemical fractionation of the vitamin B complex. To satisfactorily assay the fractions it became necessary to use both

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pigeons and rats as test animals and to take special precautions in the purification of the basal diets of these animals, especially the protein components. We also sought as an aid in B_1 assay a source of vitamin B_2 (G) which should be completely free of B_1 . The results of our studies of autoclaved yeast as a B_2 source have already appeared (6). In spite of the development of a B_1 -free autoclaved yeast as a source of vitamin B_2 , variations in animal behavior still developed.

We have sought for the cause of these variations in the methods used for purifying casein or other components of the basal diets or by utilizing other admitted sources of B_1 and B_2 . Out of the many series of tests involved we wish to report here a series that leads us in common with the investigators already cited (2-5), to question whether the B_2 -free diet resulting in rat dermatitis owes its action to lack of a factor which is identical with that preventive of human pellagra.

For our study of this series we enlisted the cooperation of Dr. James Denton, who has been identified with the study of the histology of human pellagra and black tongue of dogs (7, 8). Denton observed rarefaction of the corium, dilation of the vessels, and considerable reaction about the vessels in early cases of pellagra.¹ In all cases this was precedent to the later skin changes. Similar changes were found to take place in "black tongue." There is a corresponding fragmentation of the fibrillar material contiguous to the basal layer with very little change at this time in the epithelium itself.

His viewpoint may be stated as follows: "The distinctive lesions of pellagra and those of black tongue of dogs appear to have their origin in a failure on the part of the organism to maintain the specialized supporting tissues of epithelium in various situations." (8).

Dr. Denton separated our specimens into two distinct types of dermatitis. One he found to show the characteristic corium changes of human pellagra. The other showed the type of dermatitis that seems to always develop sooner or later on deficiency of vitamin B_2 but these animals failed to show the corium histology of early human

¹ For purposes of comparison with the photomicrographs to be discussed particular reference is made to the following photomicrographs: *J. Trop. Med.*, 1925, 5, 182, Fig. 1; *Am. J. Path.* 1928, 4, Plate 80.

pellagic skin. We present in evidence of these differences photographs of sections made for us by Dr. Denton together with data on the diets associated with the observed effects. (See Figs. 1 to 6.)

Experiment 1.—For skin see Fig. 2; for growth curve see Chart 1.

Diet: Basal.—18 per cent raw egg white protein (commercial frozen); 68 per cent purified corn-starch; 8 per cent butter fat; 2 per cent cod liver oil; and 4 per cent Osborne and Mendel salt mixture.

Vitamin B₁.—Supplied by 20 mg. daily of fullers' earth activated by the method of Williams and Waterman (9).

Vitamin B₂ (G).—Was supplied by feeding daily 0.5 gm. Liebig beef extract per rat. That this dosage provided B₂ adequate for growth effect is shown by the growth of this animal (Chart 1). After 60 to 70 days on this diet two of the three rats on the diet developed a dermatitis, viz. sore mouth, extremely red and sore paws, loss of hair on legs and chest and around joints, and to a lesser extent on the back. Nose and mouth were very sore, the eyelids and genitals inflamed, and autopsy revealed enlargement of the testes. Section of the skin of the paw of Rat 4325 is shown and reveals corium changes similar to those in human and dog pellagra. We have then in this exhibit a corium degeneration closely simulating human pellagra in spite of a dosage of vitamin B₂ in beef extract sufficient to produce excellent growth. The dermatitis of the other two rats in this series failed to clear up when the egg white protein was supplemented by 1 gm. of alkali-extracted casein daily or when 0.5 gm. daily of neutral autoclaved yeast was substituted for the beef extract.

Experiment 2.—For skin see Fig. 3; for growth curve see Chart 1.

Diet: Basal.—18 per cent extracted casein; 68 per cent purified corn-starch; 8 per cent butter fat; 2 per cent cod liver oil; and 4 per cent Osborne and Mendel salt mixture.

Vitamin B₁.—Supplied by 0.15 mg. daily of Jansen-Donath fraction 16 prepared by the method of Williams, Waterman, and Gurin (10).

Vitamin B₂.—Was supplied as a water-diffusate of beef extract. After 35 days this rat, in spite of fair growth (1.1 gm. daily), developed sore nose, sore reddened area about the mouth, bleeding at the joints, loss of hair around the eyes, and crinkled ears. The other three rats in this series also grew well but did not develop dermatitis. It must also be said that a large number of other rats have been reared on this same diet with fair growth and without dermatitis when the B₂ has been supplied by whole beef extract instead of the water diffusate. Since, however, only one of the four on the diffusate developed the skin lesions it is doubtful whether the use of the diffusate rather than whole extract as B₂ source could have been the significant factor in this result.

The section is from the skin of the chest. Like Rat 4325 it shows a true degeneration of the superficial supporting tissues in the corium. The lesion was not infected and resembled an early skin lesion of a human pellagra. Again we had

obtained a corium degeneration and dermatitis similar to human pellagra on a diet which produced good growth and was supposedly therefore adequate in B₂.

Experiment 3.—For skin see Fig. 4.

Diet: Basal.—18 per cent acid-extracted casein; 68 per cent purified cornstarch; 10 per cent cottonseed oil; 4 to 5 drops daily of cod liver oil; 4 per cent Osborne and Mendel salt mixture.

Vitamin B₁.—Supplied as for Rat 4630 by 0.15 mg. daily of Jansen-Donath fraction 16.

Vitamin B₂.—No source given.

Two rats in this series developed, after 70 to 80 days, extreme soreness of nose and around the lower part of mouth, dermatitis of the paws, loss of hair about the joints and around the eyes, and crinkled ears. Superficially these symptoms resembled closely those of Rats 4325 and 4630. Death occurred in two animals within 2 weeks after onset of symptoms. The remaining animal held apparently normal as to skin for 20 weeks, after which nose and throat soreness developed but became no worse, and the animal was killed after 27 weeks on the diet (4342).

The skin section of Rat 4343 from this series is shown. It exhibits simple atrophic thinning of the epidermis with squamae on the surface, but with no such significant corium changes as are shown by Rats 4325 and 4630. The lesions differ markedly from those of the human pellagrin.

That this rat lacked growth-promoting vitamin B₂ was evidenced by his failure to grow.

Experiment 4.—For skin see Figs. 5 and 6.

Diet: Basal.—18 per cent acid-extracted casein; 68 per cent purified cornstarch; 8 per cent butter fat; 2 per cent cod liver oil; and 4 per cent Osborne and Mendel salt mixture.

Vitamin B₁.—Supplied by Jansen-Donath fraction 16, 0.15 mg. daily.

Vitamin B₂.—No source given.

Dermatitic symptoms similar in external appearance to those of Rats 4343, 4325, and 4630 appeared in three of the four rats on the diet in about 50 days. The rats were killed on the 85th day on the diet. No growth resulted with this series, demonstrating lack of growth-promoting vitamin B₂.

Like Rat 4343 the skin sections² show no corium changes comparable to the early lesions of the human pellagrin or to those of black tongue. Some scarring of the superficial corium is observed.

Summary of Cases

The outstanding feature of these cases is the production in rats of two histologically different types of dermatitis; the appearance in one

² In all cases care was taken when selecting skin for sectioning to avoid areas where secondary infection might have occurred.

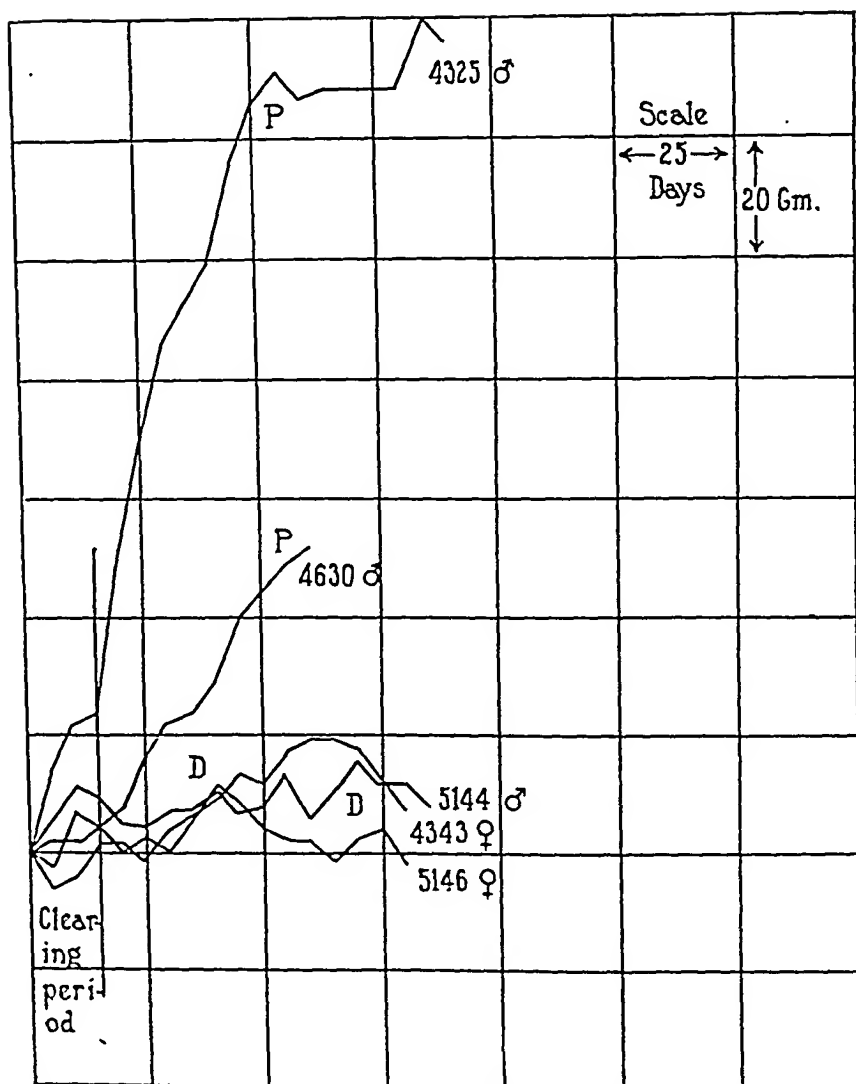


CHART 1. Growth curves of rats used in the tests reported. See pages 423 to 424.

P signifies pellagra symptoms.

D signifies dermatitis symptoms.

TABLE I

Dietary Factors Involved in the Rat Series Reported in This Article

Rats.....	4325	4630	4343	5143 and 5146
<i>Diets</i>				
Protein.....	18% egg white	18% acid-extracted casein	18% acid-extracted casein	18% acid-extracted casein
Carbohydrate....	68% corn-starch	68% corn-starch	68% corn-starch	68% corn-starch
Fat.....	8% butter	8% butter	10% cotton-seed oil	8% butter
Vitamin D.....	2% cod liver oil	2% cod liver oil	4 drops cod liver oil daily	2% cod liver oil
Salts.....	4% O & M mixture	4% O & M mixture	4% O & M mixture	4% O & M mixture
B ₁ source.....	Activated ful- lers' earth	0.15 mg. J-D 16	0.15 mg. J-D 16	0.15 mg. J-D 16
B ₂ (G) source....	Liebig beef ex- tract (0.5 gm.) and au- toclaved yeast	Water diffusate beef extract	None	None
Growth.....	Good	Good	Maintenance only	Maintenance only
Diagnosis.....	Dermatitis al- lied to hu- man pellagra according to Denton	Dermatitis sim- ulating hu- man pellagra	Dermatitis lack- ing corium picture of human pel- lagra	Dermatitis lack- ing corium changes of human pel- lagra

Acid-extracted casein was prepared as follows: 10 kilos of casein (Merck technical) was stirred into 50 liters of 20 per cent methyl alcohol solution made 0.05 per cent acid with glacial acetic acid. The mixture was stirred 24 hours by an electrically driven stirrer, allowed to stand 4 to 5 hours, and the supernatant liquid siphoned off. An equal volume of solvent was again added and procedure repeated three more times, a total of four extractions involving 96 hours of continuous stirring. The casein was then filtered on Buchner funnels, washed by stirring 20 to 30 minutes with 2 to 2½ gallons of 95 per cent ethyl alcohol, filtered, air dried, and baked at 90-100°C. for 10 hours.

The corn-starch used in the first experiments was extracted by stirring 5 hours with a large volume of 65 per cent methyl alcohol at 50°C. Three extractions of at least 5 hours each were made, the starch being filtered after each extraction, washed finally with 95 per cent ethyl alcohol, and dried in a vacuum oven at 40-50°C. until all alcohol was removed. Later this step was abandoned as no effect on the results was observable from the use of the corn-starch as purchased.

The O & M salt mixture is the classic salt mixture of Osborne and Mendel.

group of a type markedly similar to that observed in human pellagrins in spite of a diet rich in classic vitamin B₂ sources, as evidenced by growth curves, and a quite different pathological picture in the other group which was deprived of vitamin B₂ source entirely.

Others have observed dermatitis in the presence of vitamin B₂ in the diet and its failure to appear in cases where B₂ was omitted. Our data supply we believe new histological evidence that vitamin B₂ deficiency or adequacy as measured by growth effect is not alone sufficient to explain the presence or absence of true pellagra.

DISCUSSION

The view that vitamin B₂(G) may not be the pellagra-preventing vitamin has been suggested by other investigators.

In a detailed study of the distribution of vitamin B₂ in foodstuffs, Aykroyd and Roscoe (11) in 1929 advanced evidence strongly supporting the idea of its identity with Goldberger's pellagra-preventive substance. But, in 1930, Aykroyd (2) from his studies of B₂ distribution in cereals and especially in corn (maize) is led to observe: "At present the association, if any, of vitamin B₂ and pellagra is obscure."

Very recently Bliss (4) notes that Goldberger's view of vitamin B₂ as the pellagra-preventive substance has: "made less of an impression upon clinicians and laboratory workers in the South who are in actual contact with the disease than might be inferred from its ready acceptance in the standard texts today." He goes on to develop the theory that the pellagra-preventive factor is not a vitamin but instead something which supplies iron deficiency. He reports a study of the response of 51 human pellagra cases to iron therapy and the cure of black tongue by intravenous iron treatment.

Sure, Smith, and Kik (5) have reported severe dermatitis accompanied by remarkable growth and have suggested that: "the growth-promoting and anti-dermatitis factors associated with the so-called antipellagric vitamin G are not synonymous." They have supplemented this report by several others in one of which they describe an anemia in vitamin G dermatitis which developed in spite of abundance of ferric citrate in the diet.

The activated fullers' earth used in one series was prepared by the method of Williams and Waterman and shown by growth tests to be practically B₂-free (9). Later we used a fraction of high vitamin B₁ concentration obtained in Williams, Waterman, and Gurin's (10) duplication of the Jansen-Donath procedure. It represents Jansen and Donath's PtCl₄ precipitate after removal of the platinum.

The egg white we used was commercial frozen raw egg white. Solids were determined and the egg white fed to provide 18 per cent egg protein in the diet.

All of these observations as well as our own experience indicate that we cannot accept the vitamin B₂ or G, as at present defined, as identical with Goldberger's pellagra-preventive substance. Whether it is a factor in the syndrome of pellagra, and what other factors are involved, are matters demanding more experimental research and comparison of results. Whether Denton's criterion for differentiating between true pellagra and non-pellagrous dermatitis is accepted or not is also immaterial, but it is at least of interest as an attempt to define sharply the pathology of pellagra.

In Table I is given the summary of the dietary factors involved in the rat series presented in this article together with data on the method of purification of the ingredients employed. Other workers may find these data of value in comparing our tests with their own. Growth curves of the rats are shown on Chart 1.

A comparison of the dietary ingredients of Rats 4325 and 4630 with that of Rats 4343, 5143, and 5146 makes it improbable that the difference in effects could have been due to either the source of vitamin B₁ used, or to the protein, fat, carbohydrate, or salt sources used. Neither do any of the diets appear to be lacking in iron.

SUMMARY

1. Isolated cases of a dermatitis resembling histologically that of human pellagra have occurred in rats supplied with sufficient vitamin B₂ (G) in the form of beef extract or neutral autoclaved yeast to produce good growth.

2. Other rats on basal diets containing similarly prepared nutrients but deprived of any known source of vitamin B₂ (G) develop a dermatitis similar in appearance to that described by other workers, but this skin effect differs in histological picture from that found in human pellagra or in black tongue of dogs. These rats showed growth failure which supports the view that they lacked growth-promoting vitamin B₂ (G).

3. It is suggested that dermatitis in rats may be of diverse type; one resulting from vitamin B₂ (G) deficiency quite different histologically from human pellagra, and one closely allied to human pellagra and black tongue in dogs due to lack of some at present unidentified factor.

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EXPLANATION OF PLATES

PLATE 40

FIG. 1. Section of the skin from foreleg of a normal rat for comparison with the coria of the pathological specimens. Note especially the intact fibrillar collagen bundles in this section.

FIG. 2. Skin from cheek of Rat 4325.

FIG. 3. Skin from cheek of Rat 4360.

PLATE 41

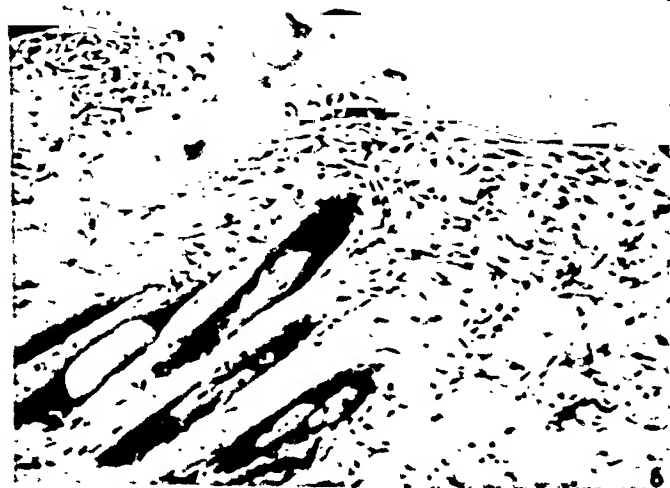
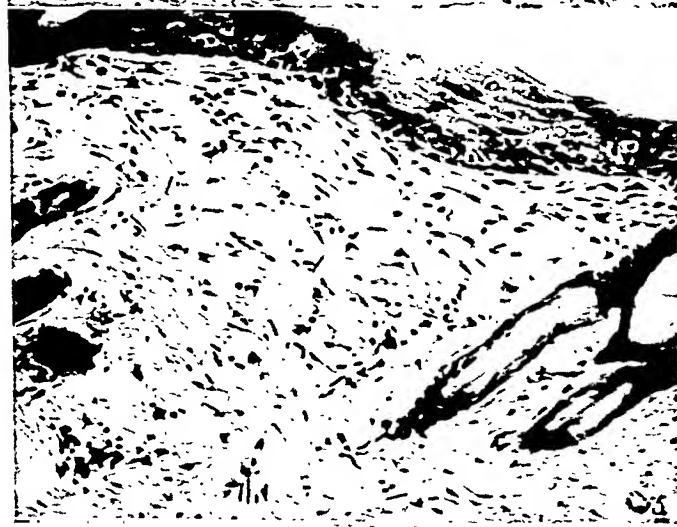
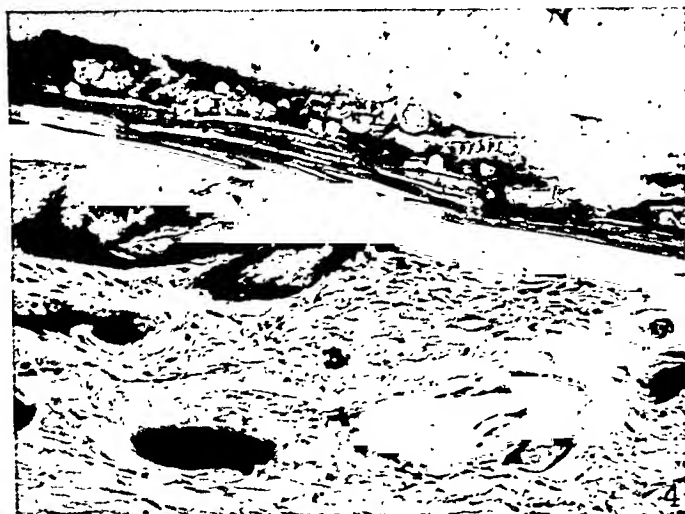
FIG. 4. Skin from forearm of Rat 4343.

FIG. 5. Skin from forearm of Rat 5143.

FIG. 6. Skin from forearm of Rat 5148.

Giemsa stain used. Magnification = 110.





CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

IV. THE SYNTHESIS OF THE *p*-AMINOBENZYL ETHER OF THE SOLUBLE SPECIFIC SUBSTANCE OF TYPE III PNEUMOCOCCUS AND ITS COUPLING WITH PROTEIN

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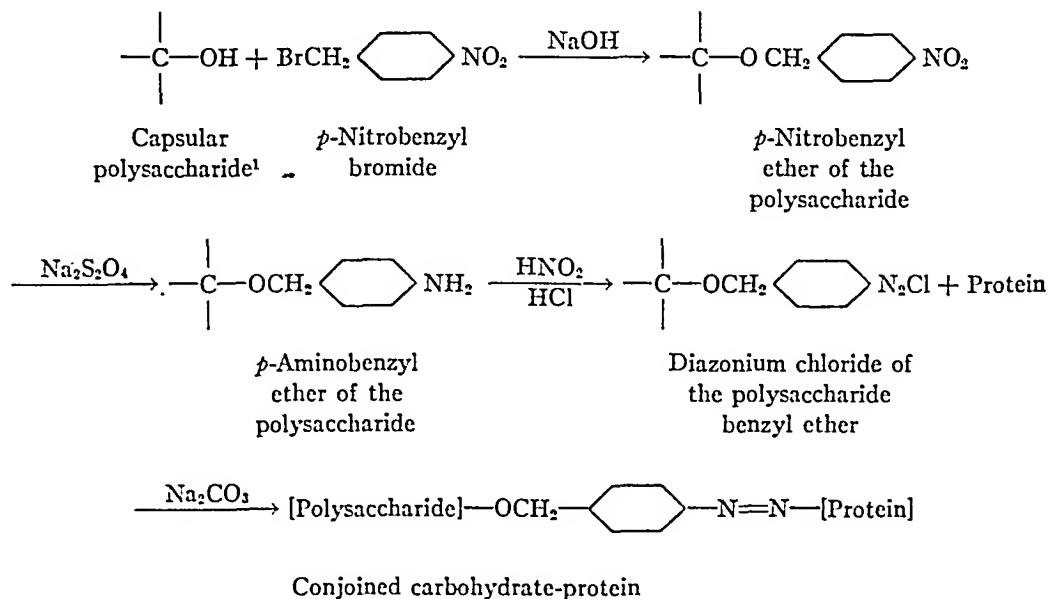
The immunological rôle of specific polysaccharides from encapsulated microorganisms has been discussed in previous communications from this laboratory (1). The capsular carbohydrate of Type III Pneumococcus may be regarded as a pure chemical entity, a polysaccharide free from nitrogenous impurities, retaining the immunological property of reacting specifically in high dilutions with homologous antiserum, yet incapable of inciting the formation of type-specific immune bodies when injected into rabbits. It has been shown, however, that simple carbohydrates when combined with proteins (2) can give rise to specific antibodies. The specificity of the antibodies thus induced is dependent upon the chemical constitution of the carbohydrate irrespective of the protein to which it is bound.

It occurred to us that if the pneumococcus polysaccharide could be combined with a foreign protein it should be possible to produce a conjoined carbohydrate-protein antigen capable of stimulating the formation of type-specific pneumococcus antibodies in the animal body. Provided the specificity of the original carbohydrate has not been too greatly altered either through chemical manipulation, or through the introduction of new molecular groupings, one should obtain on immunization with such a "synthetic antigen," antibodies which would be identical in specific action with those produced by immunization with the intact bacterial cells.

The capsular polysaccharide of Type III Pneumococcus has been shown to be a polymer of an aldobionic acid (3). The carbohydrate

itself has an acid equivalent of 338, *i.e.* it has one free carboxyl (COOH) group for every two sugar molecules. In addition to the free carboxyl group there are three free hydroxyl groups (OH) per unit of aldobionic acid in the polymeric form. If the hydrogen atom of one of these three hydroxyl groups can be replaced by a nitrobenzyl group without incurring a loss in specificity of the polysaccharide, the nitro derivative could be reduced to the amino compound which in turn might be coupled through its diazonium derivative to a protein, yielding a conjoined carbohydrate-protein. Such a complex would have only one constituent common to the pneumococcus cell, namely, the capsular polysaccharide.

The synthesis may be diagrammatically represented by the following series of reactions:



In the following account a description is given of the experimental procedure used in the chemical synthesis of the *p*-nitro and *p*-aminobenzyl ethers of the specific carbohydrate of Type III Pneumococcus

¹ Since it is impossible to draw a complete formula for the capsular polysaccharide, the latter has been represented by drawing the one carbon atom bearing a free hydroxyl group, which enters into chemical reaction with *p*-nitrobenzyl bromide in the presence of sodium hydroxide.

and the coupling of the amino derivative to serum globulin. The immunological specificity of this conjoined carbohydrate-protein complex is discussed in an accompanying publication.

EXPERIMENTAL

1. Preparation of the p-Nitrobenzyl Ether of Pneumococcus Type III Soluble Specific Substance

The nitrobenzyl ether of the carbohydrate was prepared by a method similar to that employed by Gomberg (4) in the preparation of the benzyl ethers of various carbohydrate derivatives.

1 gm. of the nitrogen-free Type III pneumococcus polysaccharide was suspended in 35 cc. of water and brought into solution by neutralizing with N/1 sodium hydroxide. To the solution was added 4.7 gm. of finely pulverized *p*-nitrobenzyl bromide. The mixture was heated to 100°C. and vigorously stirred. The nitrobenzyl bromide melted to form an oil which could be fairly well emulsified in the solution by violent stirring. 2.9 cc. of 30 per cent sodium hydroxide were added drop by drop. The rate of addition of the alkali was such that at no time were there more than a few drops in excess. The reaction was complete after $\frac{1}{2}$ hour of heating and stirring, *i.e.*, the theoretical quantity of alkali had been added to neutralize the hydrobromic acid from the *p*-nitrobenzyl bromide, leaving a neutral solution. The contents of the tube was now distilled with steam to rid the reaction mixture of *p*-nitrobenzyl alcohol. The product remaining in the flask, a yellow paste, was cooled to 0°C. and was acidified by the addition of hydrochloric acid. The product was filtered on a hardened paper, washed with small portions of ice water, and finally was dried in a vacuum desiccator.

The dry material was pulverized in a mortar and extracted with acetone in a Soxhlet extractor. A quantity of soluble colored material,—probably condensation products from the *p*-nitrobenzyl bromide, was thus eliminated. The polysaccharide-ether remaining in the extraction thimble was dissolved in alkali, made up to 200 cc. with water, cooled to 0°C., and acidified by the addition of hydrochloric acid. The precipitate (the nitrobenzyl ether of the soluble specific substance) was centrifuged and then reprecipitated. The final product was filtered on a hardened paper and was then dried in a desiccator. About 1.1 gm. of material were recovered.

The nitrobenzyl ether of the soluble specific substance of *Pneumococcus* was thus obtained as a pale yellow compound, soluble in dilute alkali by virtue of the free carboxyl group in the molecule. The compound itself is insoluble in water, though its sodium salt is readily soluble. The compound may be precipitated by adding acid to an

aqueous solution of its sodium salt. The ether does not dissolve in the usual organic solvents. It is soluble in 75 per cent aqueous acetone, and is slightly soluble in 50 per cent alcohol.

The compound has a specific optical rotation of -26.50° , an acid equivalent of 480 (the calculated value for a mononitrobenzyl ether of a polymer of the aldobionic acid $C_{11}H_{19}O_{10}COOH = 473$), and a nitrogen content of 2.99 per cent (calculated value = 2.96 per cent). The substance reacts specifically with Type III antipneumococcus serum in dilutions of 1:5,000,000.

2. Preparation of the Aminobenzyl Ether of the Soluble Specific Substance of Type III Pneumococcus

1 gm. of the nitrobenzyl ether of the soluble substance was suspended in 20 cc. of water. The substance was brought into solution by neutralization with 20 per cent sodium hydroxide. The solution was warmed to $50^\circ C.$ and to it was slowly added a freshly prepared saturated solution of sodium hydrosulfite. The solution was kept near the neutral point after each addition of hydrosulfite, by the cautious addition of alkali. When a slight excess of the reducing agent persisted, as determined by the ability of the reaction mixture to bleach litmus paper, the solution was cooled in a freezing mixture and then carefully acidified with hydrochloric acid (sp. gr. 1.09). A yellow precipitate of the amino polysaccharide-ether separated out. After centrifugation, it was dissolved, and the solution was dialyzed against successive changes of distilled water until free of chlorides and sulfates. The solution was then poured into 25 volumes of chilled acetone, and after it had flocked out of solution, the amino ether was filtered on a hardened paper. About 0.8 gm. was recovered.

The aminobenzyl ether of the Type III soluble specific substance was obtained as a yellow ash-free amorphous powder soluble in warm water, and in aqueous acetone (75 per cent). It has an optical rotation of -28.5° , an acid equivalent of 453 (calculated = 443.2), and a nitrogen content of 3.15 per cent (calculated = 3.16 per cent). The substance reacts with Type III antipneumococcus serum in dilutions of 1:5,000,000. When weighed, dried samples of this amino compound are dissolved in water, and then 2.2 equivalents of hydrochloric acid added, and the solution titrated at $0^\circ C.$ with $N/20$ sodium nitrite, it is found that the derivative uses up exactly the theoretical quantity (calculated on the nitrogen basis) to form the diazonium derivative. Our first few preparations of the amino ether utilized only some 75

per cent of the theoretical quantity of nitrite when titrated. Later preparations, however, appeared to have all of the nitrogen in the amino form, for they utilized the theoretical quantity of nitrous acid.

3. The Coupling of the Aminobenzyl Ether of the Soluble Specific Substance to Serum Globulin

250 mg. of the aminobenzyl ether soluble specific substance were dissolved in 25 cc. of water. The solution was cooled to 0°C. and to it was added 2.2 mols of normal hydrochloric acid. To the opalescent solution was now added 1 mol of N/10 sodium nitrite. The solution was stirred for 20 minutes. Normal sodium hydroxide was then added *very* cautiously until the solution was just neutral to litmus paper. The solution of diazotized soluble substance was now poured into an alkaline solution of serum globulin, (prepared from normal horse serum by repeated precipitation with half saturation of ammonium sulfate) containing 800 mg. of the latter dissolved in 30 cc. of N/2 sodium carbonate at 0°C. An orange color soon developed which deepened on standing, until finally, after 2 hours standing at 0°C., the presence of free diazonium body could no longer be demonstrated. The deeply colored solution was now acidified carefully by the addition of 10 per cent trichloroacetic acid. A yellow precipitate separated, which was centrifuged at low speed. The supernatant liquid still contained relatively large quantities of uncombined protein, and some free soluble substance. It was discarded.

The precipitate was suspended in 30 cc. of salt solution and was briskly stirred for 30 minutes to break up any lumps which were present. As soon as a uniform suspension of the yellow precipitate was obtained, it was dissolved by the addition of N/10 sodium hydroxide. The fine particles swelled and gave what was apparently a solution, but a large amount of transparent orange jelly could be centrifuged away from some of the true solution of the substance. However, instead of centrifuging and discarding this jelly, the entire suspension was again acidified with a small amount of trichloroacetic acid. The precipitate thus formed was again centrifuged and the clear supernatant liquid was tested for the presence of specifically reacting polysaccharide. Solution and reprecipitation were repeated until no more reactive polysaccharide could be found in the supernatant liquid. This required, in all, about three precipitations.

The final precipitate was now suspended in 30 cc. of 0.9 per cent salt solution containing 0.25 per cent tricresol; N/10 sodium hydroxide was added until the mixture was very faintly alkaline to litmus, but not alkaline to phenolphthalein. Most of the compound went into solution, a small amount still remained in suspension as a jelly-like conglomerate. The mixture was diluted to 150 cc. with 0.25 per cent tricresol salt solution and was used for immunization purposes.

The conjoined carbohydrate-protein reacted with Type III anti-pneumococcus serum in dilutions of 1:500,000. When the precipi-

tate thus formed was separated by centrifugation, it was found to be colored yellow, and when it was dissolved in alkali, an orange solution was obtained. This was an indication that the specific polysaccharide actually is bound to the protein by way of the chromophoric linkage —N=N— and that when the specific part of the protein-carbohydrate complex reacts with pneumococcus antibody, the complex precipitates *in toto*. The carbohydrate-protein was found to contain 13 per cent of sugar, calculated as glucose. This is in fair agreement with the amount of bound polysaccharide calculated from the reactivity of the carbohydrate derivative with pneumococcus antiserum, *i.e.* roughly 10 per cent, since the sensitivity of this reaction is 1:5,000,000. This polysaccharide-protein complex has been used to immunize animals. The results of the immunological studies are presented in the following paper.

SUMMARY

1. The *p*-amino and *p*-nitromonobenzyl ethers of the specific polysaccharide of Type III Pneumococcus have been prepared.

2. The diazonium ether of the specific polysaccharide has been coupled with serum globulin to yield a specific polysaccharide-protein complex and this complex has been used for immunization. The results of the immunological studies are presented in the following paper.

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CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED
CARBOHYDRATE-PROTEINSV. THE IMMUNOLOGICAL SPECIFICITY OF AN ANTIGEN PREPARED BY
COMBINING THE CAPSULAR POLYSACCHARIDE OF TYPE
III PNEUMOCOCCUS WITH FOREIGN PROTEIN

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The fundamental studies of Landsteiner and his coworkers (1) on complex antigens have established the important principle that the introduction into the protein molecule of a simple non-protein radical confers a new immunological specificity on the antigenic compound. Furthermore, this newly acquired specificity has been shown to depend upon the nature of the new chemical grouping thus introduced.

Previous studies from this laboratory (2) on the chemo-immunological properties of "synthetic antigens," prepared by combining a simple carbohydrate radical with protein, have shown that the specificity of the newly formed compounds is determined in each instance by the chemical individuality of the reactive carbohydrate, irrespective of the protein to which it is attached. Simple derivatives of glucose and galactose, which by themselves are non-antigenic will, when coupled to a common protein, stimulate the formation of antibodies that are specific for the particular sugar used. Antisera, produced by immunization with the conjugated sugar-proteins, invariably reflect the controlling influence of the carbohydrate on the specificity of the whole antigen. So sensitive is this chemo-specific effect, that mere differences in the spatial arrangement of the groups on a single carbon atom in two glucosides otherwise identical, were found sufficient to change completely the antigenic specificity of the respective compounds.

In order to test the possibility of synthesizing a specific antigen by

combining a bacterial carbohydrate with a foreign protein, the specific capsular polysaccharide of Type III *Pneumococcus* was purposely chosen, since in its purified form it contains no nitrogen and may be regarded as a definite chemical entity. Further, if results were obtained by the use of this particular bacterial polysaccharide, they would be the more significant, since the isolated pure substance alone has never been found to elicit antibodies in rabbits, and even the intact bacterial cells from which it is derived commonly fail to incite the formation of type-specific antibodies in these animals.

From a chemical point of view, the difficulty lay in synthesizing the appropriate derivative of the capsular polysaccharide. It must be one capable of coupling with protein on diazotization and one in which the chemo-specific groups of the bacterial sugar are not masked or destroyed in its preparation. The details of the chemical methods used in the synthesis of the aminobenzyl ether of the capsular polysaccharide of Type III *Pneumococcus* have been described in the preceding paper (3). The diazonium derivative of the polysaccharide was coupled with globulin prepared from horse serum, and the newly formed compound was tested for antigenicity by repeated intravenous injection in rabbits. The sera of the immunized animals were tested for the presence of type-specific agglutinins and protective antibodies against encapsulated, virulent strains of Type III *Pneumococcus* and for precipitins against the type-specific capsular polysaccharide and its aminobenzyl derivative. The treated rabbits were subsequently infected with a rabbit-virulent strain of Type III *Pneumococcus* to determine whether, as a result of immunization, they had acquired active immunity against Type III infection.

In evaluating the immunological findings presented in this paper, the fact should be borne in mind that the antigen used in the experiments has in common with the Type III *Pneumococcus* only the specific capsular polysaccharide, and that the protein with which it is conjugated is of widely remote biological origin.

EXPERIMENTAL

Methods

Immunization.—Rabbits were immunized by the intravenous injection of from 1 to 2 cc. of solutions of the conjugated carbohydrate-protein antigen daily for

six doses, and the course of injections was repeated a second time after a rest period of 7 days. 8 days after the last injection the rabbits were bled and the serum tested for type-specific pneumococcus antibodies.

Antigen.—The antigen, prepared as described in the preceding paper, was preserved by the addition of 0.25 per cent tricresol. Each preparation was standardized on the basis of nitrogen content, so that 1 cc. of the solution contained 5 mg. of protein. This method, however, does not indicate the amount of bound carbohydrate and hence is not an accurate measure of the effective antigenic complex. The protein, to which the aminobenzyl ether derivative of Type III polysaccharide was bound by the diazo reaction, was globulin prepared from horse serum by precipitation with ammonium sulfate.

Technic of Immunity Reactions.—In the precipitin reactions, the immune serum was diluted in the proportion of two parts of serum to three of salt solution and 0.5 cc. of this dilution containing 0.2 cc. of original serum was added to 0.5 cc. of varying dilutions of reacting substance. The final concentration of precipitinogen in the reaction mixture is shown in the protocols. The protection tests were done by the usual technic; mice were injected intraperitoneally with 0.2 cc. immune serum together with varying dilutions of an actively growing culture of *Pneumococcus*. The dilutions were so made that the total volume injected was 1 cc. in all instances.

I. Type-Specific Antipneumococcus Antibodies

1. *Precipitins.*—The sera of rabbits immunized with the Type III "synthetic antigen" were tested for the presence of precipitins for the original capsular polysaccharide and for the aminobenzyl ether derivative used in preparing the antigen. The introduction of the para-aminobenzyl radical into the polysaccharide molecule did not destroy the immunological specificity of the derivative, since the latter reacted with Type III antipneumococcus horse serum in high dilutions, showing a specific reactivity comparable to that of the original polysaccharide from which it was derived. It was of interest, therefore, to determine whether this specific carbohydrate derivative, when bound with protein, would stimulate the formation of precipitins reactive not only with the amino derivative but also with the polysaccharide itself, when both substances were used in the free, soluble form.

The precipitin reactions of immune rabbit serum with the original and modified form of the Type III capsular polysaccharide are compared in Table I. The results show that the serum of a rabbit immunized with the "synthetic antigen" reacts with the native polysaccharide and with its aminobenzyl ether in equally high dilutions.

Similarly, as previously pointed out, the serum of a horse immunized with the intact bacterial cells containing the natural Type III antigen precipitates the polysaccharide derivative as well as it does the polysaccharide itself. The evidence is, therefore, that the specifically reactive groups of the polysaccharide have not been chemically masked in forming the new derivative, and that when this specifically reactive carbohydrate is chemically bound with protein to form a new antigen, it orients the immune response as specifically as does the polysaccharide itself in the form in which it exists as a natural antigen in the encapsulated cells.

TABLE I

Precipitins for the Capsular Polysaccharide of Type III Pneumococcus in Serum of Rabbits Immunized with Type III Synthetic Antigen

Dilution of carbohydrate	Purified preparation of Type III polysaccharide	Aminobenzyl ether of Type III polysaccharide
1:100,000	++±	+++
1:200,000	+++	+++
1:400,000	++±	+++
1:800,000	+±	+
1:1,600,000	+	±

+++ = complete precipitation with compact disc.

++ = diffuse turbidity.

+ = slight turbidity.

2. *Agglutinins*.—The sera of rabbits, prepared by serial injections of the chemically combined antigen, specifically agglutinate encapsulated strains of Type III pneumococci.

The results of the agglutination reactions, as illustrated in Table II, show that while the titre of agglutinins is not high, the serum is type-specific in that it agglutinates only Type III pneumococci, and fails to react with organisms of Type I and Type II. The fact that the immune serum does not agglutinate the non-encapsulated R forms of *Pneumococcus* was to be expected, since the antigen contains none of the somatic constituents commonly present in all pneumococci. The lack of these cellular substances in the artificial antigen is reflected in the serum by the absence of the species-specific antibodies, which

are ordinarily present when the whole cell is used as the immunizing agent.

TABLE II

Specificity of Agglutination Reaction with Serum of Rabbit Immunized with Type III Synthetic Antigen

Pneumococcus	Immune serum			
	1:5	1:10	1:20	1:40
Type I.....	—	—	—	—
Type II.....	—	—	—	—
Type III.....	++++	+++	+	±
"R" strain*.....	—	—	—	—

++++ = complete agglutination with disc formation.

— = no reaction.

* Derived from Pneumococcus Type II.

TABLE III

Protective Action in Mice of Sera of Rabbits Immunized with Type III Synthetic Antigen

Pneumococcus Type III — cc.	Immune rabbit sera (0.2 cc.)				Virulence controls No serum	
	(a)	(b)	(c)	(d)		
0.01	— —	— —	D 22 D 22	D 7 D 24	—	—
0.001	— —	— —	S S	S S	—	—
0.0001	S S	S S	S S	S S	—	—
0.00001	S S	S S	S S	S S	—	—
0.000001	S S	S S	S S	S S	D 42 D 42	D 42 D 42
0.0000001	— —	— —	— —	— —	D 42 D 45	D 42 D 45
0.00000001	— —	— —	— —	— —	D 75 D 45	D 75 D 45

S = survival.

D = death of animal, the numeral indicates the number of hours elapsing before death.

— = not done.

3. *Protective Antibodies.*—To ascertain whether passive immunity against pneumococcus infection could be conferred on mice by the injection of the serum of a rabbit immunized with the artificial Type

III antigen, protection tests were carried out by the technic described. A constant amount of immune serum (0.2 cc.) was injected intraperitoneally into mice together with varying quantities of a virulent culture of *Pneumococcus*.

The results given in Table III show that the immune rabbit sera were effective in protecting mice against 10^{-3} cc. of a virulent culture

TABLE IV

Type Specificity of Protective Antibodies in the Sera of Rabbits Immunized with Type III Synthetic Antigen

Immune serum	Amount of culture	Pneumococcus		
		Type I	Type II	Type III
cc.	cc.			
0.2	10^{-3}	—	—	S S
0.2	10^{-4}	D 28 D 28	D 28 D 28	S S
0.2	10^{-5}	D 46 D 28	D 28 D 46	S S
0.2	10^{-6}	D 28 D 46	D 28 D 28	S S
Virulence controls	10^{-6}	D 46	D 28	D 46
	10^{-7}	D 72	D 46	D 46
	10^{-8}	S†	D 46††	D 46†††

S = survival.

D = death of animal; the numeral indicates the number of hours elapsing before death.

— = not done.

† The number of colonies developing in blood agar seeded with this inoculum = 0.

†† The number of colonies developing in blood agar seeded with this inoculum = 3.

††† The number of colonies developing in blood agar seeded with this inoculum = 2.

of Type III *Pneumococcus*, of which 10^{-3} cc. was invariably fatal when injected alone into untreated control animals. Although the maximum degree of protection afforded by the serum was not high, the special interest of these experiments lies in the fact that the protective antibodies were produced in response to a combined antigen containing a specifically reactive carbohydrate which by itself fails to evoke any immune response in rabbits.

The specificity of the protective antibodies in the sera of rabbits immunized with the Type III synthetic antigen is shown in Table IV.

The protective action of the immune serum is strictly type-specific. Mice receiving the immune serum survived infection with Type III *Pneumococcus*, but succumbed promptly to minimum doses of Type I or Type II culture. The marked specificity of the protection afforded by the serum is further evidence that the antibodies concerned in the reaction derive their specificity from the polysaccharide component of the whole antigen. The protein constituent of the antigen appears to contribute nothing to the antibacterial properties of the serum, except in so far as it renders the carbohydrate antigenic.

4. *Active Immunity*.—Of the series of rabbits injected at various times with effective preparations of antigen, none have failed to respond with the formation of type-specific antibodies. It was of interest, therefore, to ascertain whether rabbits in the sera of which Type III *Pneumococcus* antibodies were demonstrable had acquired active immunity against infection with this organism. In order to determine this point, several of the treated animals were subsequently infected with multiple lethal doses of a virulent culture of Type III *Pneumococcus*.

11 days after the last injection of antigen, four immunized rabbits whose serum contained type-specific antibodies were infected by the intradermal injection of 0.2 cc. of an 18 hour blood broth culture of a rabbit-virulent strain of Type III *Pneumococcus*. The virulence of this strain, maintained by repeated rabbit passage, was such that 0.00001 cc. of the culture alone injected into the skin proved fatal within 72 hours.

Four rabbits, which had received repeated injections of the "synthetic antigen," were reserved and later tested for active immunity. All four of these rabbits survived infection. In the two animals which had the highest titre of type-specific antibodies in their serum, the infection ran a practically afebrile course with only a slight inflammatory reaction at the point of inoculation; in the other two rabbits, the temperature remained relatively low and the skin lesion was not severe. At no time during the course of the infection were pneumococci present in cultures of the blood of the immunized rabbits, whereas in the normal controls the bacteremia invariably increased up to the time of death.

The results demonstrate that an antigen prepared by combining the Type III capsular polysaccharide with an animal protein is capable of inducing in rabbits an active immunity against infection with a virulent culture of Type III Pneumococcus. The only constituent of Pneumococcus in the artificial antigen is the capsular polysaccharide. Since this carbohydrate alone is non-antigenic in rabbits, the antibacterial immunity induced by the combined antigen can be ascribed only to the antigenicity acquired by the polysaccharide through combination with the foreign protein. The results bring evidence that an effective, active immunity can be developed in which the only antibacterial antibodies formed are those directed against the capsular component of the pneumococcus.

II. Antiprotein Antibodies

In the conjugated antigen, the protein used in combination with the Type III capsular polysaccharide was globulin derived from normal horse serum. The total protein of the antigen was estimated by determining the nitrogen content of the various preparations. This method of standardization, while affording a measure of the total globulin present, does not indicate the amount of protein that is bound with the polysaccharide to form the effective type-specific antigen. The sera of immune rabbits invariably contained not only type-specific antibodies for Type III Pneumococcus, but also specific precipitins for the globulin of horse serum.

The presence in immune rabbit serum of precipitating antibodies for the foreign protein used in preparing the antigen is shown in Table V. The antiprotein precipitins evoked by the combined antigen may be attributed to one or both of two possibilities. In the first place, despite attempts to remove the excess of unbound globulin from solution, it is possible that sufficient protein remains free and uncombined with the polysaccharide to function independently as antigen. It is also possible, however, that even in the absence of any free globulin, the combined antigen itself may give rise to two qualitatively different antibodies, each specifically related to the corresponding component of the antigenic complex. This explanation of the concurrence of two distinct varieties of antibodies in the immune serum involves the assumption, that while the carbohydrate

has acquired antigenicity through combination with the globulin, the protein molecule itself has not lost its own specific antigenic property. According to this view, a single compound of carbohydrate and protein may possess the dual antigenic property of stimulating two separate and specific antibodies; one evoked by the newly acquired antigenicity of the polysaccharide in union with globulin, and the other stimulated by the protein molecule itself in which the chemo-specific groups essential for antigenicity have not been masked in the linkage with the carbohydrate.

TABLE V

Antiglobulin Precipitins in Sera of Rabbits Immunized with Type III Synthetic Antigen

Immune sera	Globulin (horse serum)*			
	1:20,000	1:40,000	1:80,000	1:160,000
(a)	++++	+++	++	±
(b)	++++±	+++	++	+
(c)	++++	+++±	±	±
(d)	++++	+++	±	+
(e)	++++±	++±	+	±

++++ = flocculent precipitation, clear supernatant.

+ = slight, diffuse turbidity.

* The diazonium derivative of the capsular polysaccharide of Type III Pneumococcus was coupled with globulin from horse serum.

Of the two explanations, the first, namely that the free globulin in the antigenic mixture accounts for the antiprotein antibodies, is the simpler and perhaps the more likely one. However, the second concept, which ascribes a dual antigenicity to the sugar-protein, affords an immunologically interesting and theoretically possible explanation.

DISCUSSION

In the present state of our knowledge it would be hazardous to predict the precise conditions under which complex carbohydrates by themselves may function as antigens. The present study simply defines certain experimental conditions under which the capsular polysaccharide of Type III Pneumococcus, in chemical union with a

foreign protein, is rendered specifically antigenic in a particular species of animals, in which the carbohydrate alone has never been found to incite antibody formation. The "synthetic antigen" elicits in rabbits a type-specific antipneumococcus response, which neither one of its constituents alone is capable of inciting when injected singly into these animals.

In order to effect the chemical union, it was first necessary to synthesize a derivative of the bacterial carbohydrate that could be diazotized and coupled to protein by means of the diazo linkage. This was accomplished by forming the aminobenzyl ether of the polysaccharide, a derivative which fulfills the chemical requirements while still retaining the serological specificity of the original carbohydrate.

Antisera prepared by immunization with the combined antigen contain type-specific antibodies which precipitate the Type III capsular polysaccharide, agglutinate pneumococci of the homologous type, and protect mice against infection with virulent strains of Type III *Pneumococcus*. The fact that, in the present instance, the antipneumococcus response is produced by an antigen known to contain but a single component of the bacterial cell, indicates the unity of the antibodies participating in the type-specific reactions of precipitation, agglutination, and protection, and relates the specificity of these antibodies to that of the capsular polysaccharide in the reactive part of the antigenic molecule.

CONCLUSIONS

1. Type-specific antipneumococcus immunity has been induced in rabbits by immunization with antigen prepared by combining a specific derivative of the capsular polysaccharide of Type III *Pneumococcus* with globulin from horse serum.

2. Rabbits immunized with this antigen acquire active immunity against infection with virulent Type III pneumococci.

3. The sera of the immune rabbits contain type-specific antibodies which precipitate the Type III capsular polysaccharide, agglutinate Type III pneumococci, and specifically protect mice against Type III infection.

4. The experimental data are discussed with reference to: (1) the concurrence in the immune sera of type-specific antibodies for Pneu-

mococcus and precipitins for horse globulin; (2) the determining influence of the capsular polysaccharide on the specificity of the antigen as a whole; (3) the unity of the type-specific precipitins, agglutinins, and protective antibodies induced by a single component of the pneumococcus in chemical union with an unrelated, animal protein.

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SOME ULTRAVIOLET PHOTOMICROGRAPHS OF *B. SUBTILIS*

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PLATES 42 TO 44

(Received for publication, May 29, 1931)

In the course of some studies being made with the ultraviolet microscope we have prepared photographs of *B. subtilis* which yield useful information concerning the internal structure of this bacterium and the way spores arise within it.

Two bacterial strains were examined. One was non-sporulating and came from the Type Collection in Chicago; the other, which grew larger bacilli and readily produced spores in old cultures, was isolated in this laboratory. Before being photographed the living unstained cells were transferred to the surface of a thin layer of NaCl-agar spread upon a quartz slide. In this way they were fixed in position and kept stationary during exposure.

Technique

The microscope was a modification of the instrument designed by Barnard¹ and manufactured by Beck. Its optical parts, as used in the present experiments, were, however, quartz objectives and eyepieces by Zeiss. As their design requires, these objectives have been employed with light of wave length 2750 Å. Desired objects are found with the green line of the mercury arc; approximate focus in the ultraviolet is then achieved by applying a precise but arbitrarily determined correction. The focal plane is shallower with ultraviolet than with visible light and it is usually necessary to make a series of exposures at different depths through a preparation. This has been done by taking photographs upon motion picture film. The small Leica camera modified by removing its glass optical parts and giving it a very rigid support behind the eyepiece of the microscope is convenient for this purpose. Ordinary positive film gives excellent results—it is sufficiently

¹ Barnard, J. E., *Lancet*, 1925, 2, 117.

rapid in the ultraviolet and its fine grain permits very satisfactory enlargements. The accompanying photographs have thus been made, the objective being the standard Zeiss 1.70 mm. glycerine immersion monochromat. Exposure times varied from $\frac{1}{2}$ to 3 seconds.

Ultraviolet microscopy has two outstanding advantages over that using visible light. One resides in the greater resolving power which is a consequence of the short wave length; the other arises from the fact that, since some proteins absorb in the ultraviolet more strongly than others, it is often possible to see detail in living and unstained cells. Both of these advantages are realized in the accompanying pictures. The first figure, of a 5 hour culture on agar, makes it clear that young and rapidly growing cells contain no observable structures. In older cultures of both the non-sporing and the sporing lines cells become granulated (Figs. 3-6). At first these granules are few in number and small. Very old bacilli, however, are often filled with inclusions of all sizes (Fig. 2). It is important to notice that in none of these photographs is there any indication of formed nuclei.

Especially interesting is the evidence bearing upon spore production. These bodies are intensely absorbing (Figs. 2-5) and many stages in their development can therefore be seen directly. They do not arise through the gradual accumulation and merging together of the refractile granules or of any preexisting cell bodies that have strong ultraviolet absorption. Instead, at the first appearance they have their final size and shape. In initial stages they are only faintly absorbing but as they develop this absorption becomes progressively greater (Fig. 4). Cells containing mature spores are approximately as opaque as other bacteria. This suggests that spores when fully formed are not a mere condensate of a bacterium's protoplasmic or chromatin content.

There are three frequently expressed views concerning the origin of spores.² According to one they arise after a nuclear division which has partitioned the chromatin between a spore-anlage and the cell nucleus. Other investigators have described them as resulting from the coalescence of chromatin granules which in more recent work are definitely associated with nuclei thought to be present. It is apparent

² See for example Gotschlich, E., in Kolle, W., Kraus, R., and Uhlenhuth, P., *Handbuch der pathogenen Mikroorganismen*, Jena, Gustav Fischer, and Berlin and Vienna, Urban and Schwarzenberg, 3rd edition, 1929, 1, 33.

that the spores of *B. subtilis* do not have the small beginnings required by this hypothesis. A third idea makes spores the result of a more or less gradual concentration of cell substance. Some have thought that this concentration was in the main a dehydration succeeded by the infiltration of lipoids. In its broad outlines such an hypothesis is not incompatible with our photographs. At present ultraviolet microscopy is obviously unable to interpret chemical changes that may take place but nevertheless pictures such as Fig. 4 seem to demand a mechanism more elaborate than mere dehydration.

EXPLANATION OF PLATES

PLATE 42

FIG. 1. A young culture of *B. subtilis* growing on agar, photographed 5 hours after inoculation and 10 minutes after being covered with quartz. 3300 \times .

FIG. 2. Cells from a 6 day agar culture transferred to a film of fresh nutrient agar just before being photographed. The large inclusions present in the degenerating cells can easily be seen. 2500 \times .

PLATE 43

FIG. 3. Cells of a 31 hour old culture of non-sporulating *B. subtilis* growing on agar. The inclusions present in nearly every cell are clearly visible. 3500 \times .

FIG. 4. Cells from a 20 hour culture of spore-forming *B. subtilis*. The bacteria were transferred to a film of pure agar immediately before being photographed. Different cells contain spores in all stages of development (and of corresponding opacity). 3500 \times .

PLATE 44

FIG. 5. Bacteria from a 24 hour culture of sporulating *B. subtilis* grown on agar. They were transferred before being photographed to a film of 2 per cent agar in 0.85 per cent NaCl on a quartz slide. It will be noticed in this and the preceding picture that, though spores do not grow from granules, cells containing mature or developing spores are relatively free from inclusions. 3500 \times .

FIG. 6. The same field as the preceding but with the focal plane slightly above that of the bacteria. 3500 \times .

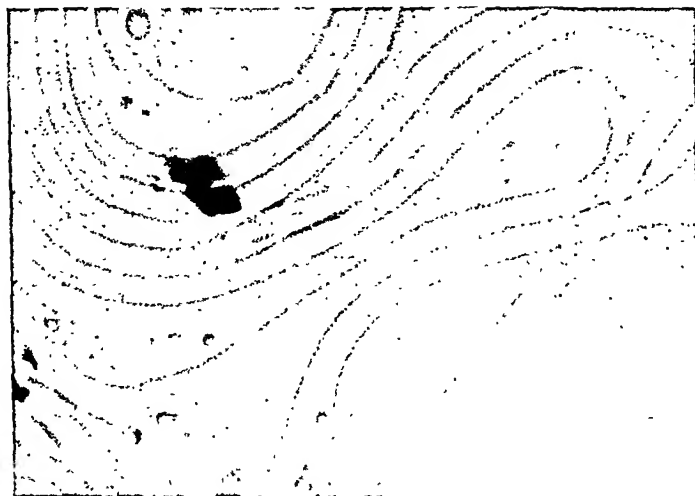


FIG. 1

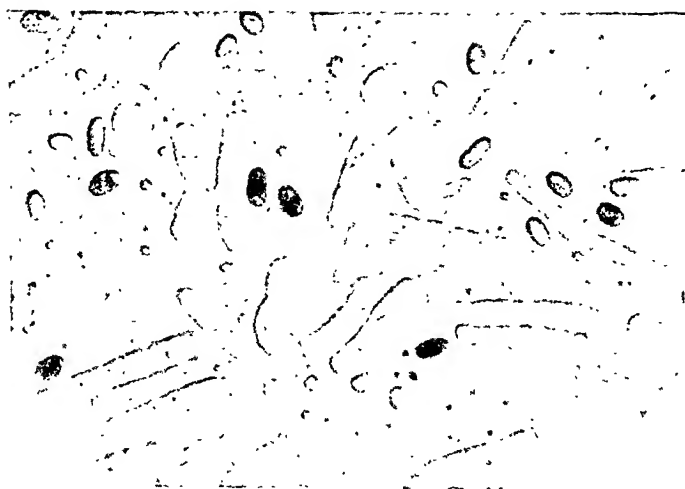


FIG. 2



§ 11. 2



§ 11. 2

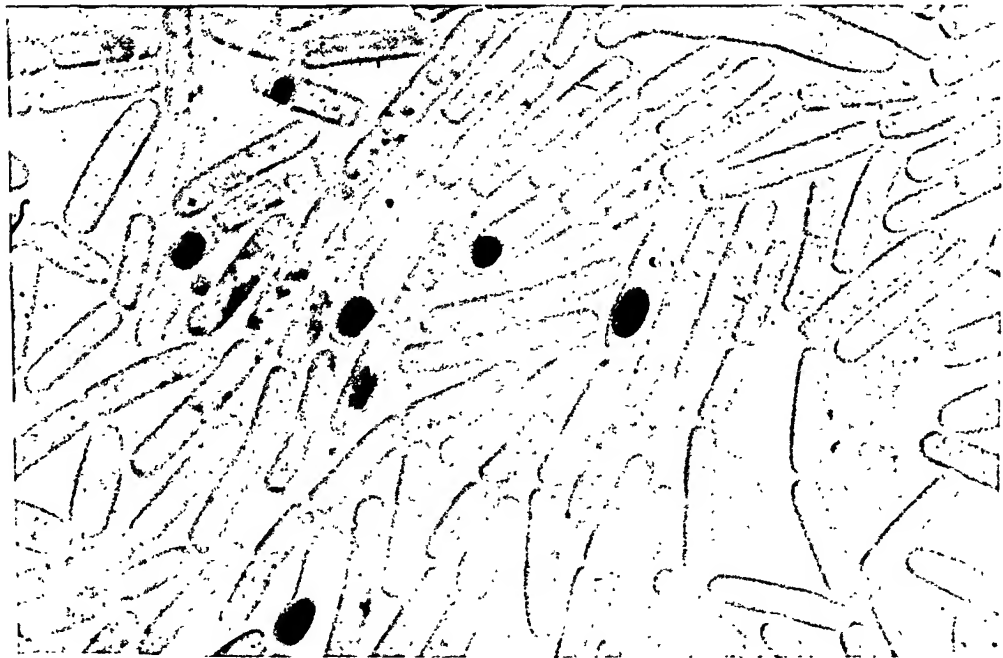


FIG. 5



FIG. 6

CULTIVATION OF VACCINE VIRUS FOR JENNERIAN PROPHYLAXIS IN MAN

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PLATES 45 TO 47

(Received for publication, June 15, 1931)

In a previous communication (1) a simple medium for the cultivation of vaccine virus was described. The results of the work reported at that time clearly indicate that vaccine virus is capable of multiplication in the presence of minced chick embryo tissue suspended in Tyrode's solution. The experiments, however, were conducted with a neurovaccine virus, and, although the active agent engendered in cultures caused typical vaccinal lesions in rabbits, it was deemed best not to test it in human beings. To obtain a culture virus for Jennerian prophylaxis in man, it seemed advisable to adapt a dermal strain of vaccine virus to our method of cultivation. The present paper deals with the results of this work.

Methods and Materials

Virus.—Vaccine virus, Lot 611, prepared by the New York City Board of Health was used to initiate the cultures. In a letter to me, the original source of the virus is described by Mr. Chas. R. Tyler.

"Regarding the history of our strain of vaccine virus: the following statement appears in the Yearly Report of the New York City Board of Health for 1874-75.

"We began vaccinating with virus of the same stock as that which had been supplied by the late Dr. Loines of the Eastern Dispensary and myself for about 5 years and which had been used and sold by him for about 20 years previously. This virus was originally obtained from England by Dr. Loines and in all probability was descended from the stock furnished by Jenner. As it always developed characteristic Jennerian vesicles and as it always thoroughly protected from smallpox those upon whom it was used, Dr. Loines never thought favorably of employing any other."

"The same strain of virus has been in use since the time of this report. The virus has been 'humanized' at various times, usually from one to three times yearly."

Tissues.—Embryonic tissue was obtained from eggs, incubated 9 to 12 days, that had been opened according to the method of Carrel and Rivers (2) or that of Eagles and McClean (3). If the embryos were contaminated by yolk, they were thoroughly washed in sterile Tyrode's solution before being minced. Furthermore, the eyes were removed, because the pigment granules contained in them often led to confusion when smears were made to determine the sterility of the cultures. Finally, the embryos were placed in a sterile watchglass contained in a Petri dish, finely minced with scissors, and then distributed in proper amounts into flasks by means of a pipette.

Tyrode's Solution.—Tyrode's solution prepared according to the following formula and sterilized by filtration was used: NaCl, 8 gm.; KCl, 0.2 gm.; CaCl₂, 0.2 gm.; MgCl₂, 0.1 gm.; NaH₂PO₄, 0.05 gm.; NaHCO₃, 1.0 gm.; glucose, 1.0 gm.; water *q.s.* 1000 cc. During the preparation and filtration of the solution, CO₂ is liberated, and the pH is found to be between 8.0 and 8.4. When living tissue is added to the solution, however, CO₂ is supplied by it and the pH becomes less alkaline reaching a point compatible with the survival of the embryonic tissue and the multiplication of the virus.

Containers.—Containers for the production of vaccine virus in large amounts must be of sufficient size and easy to handle. Furthermore, they must permit aeration and prevent contaminations and evaporation. Flasks (collar flasks) designed to meet these requirements were described in a previous paper (1) and have been found to be satisfactory for this work. Two sizes have been used, one with approximately the dimensions of a 50 cc. Erlenmeyer flask, the other with approximately the volume of a 250 cc. Erlenmeyer flask. In the small container 5 cc. of medium were placed, while in the large one 15 cc. were used. Such small amounts were placed in each flask in order to have the medium distributed as a thin layer. In case collar flasks cannot be obtained, 50 cc. and 250 cc. Erlenmeyer flasks make good substitutes. All glassware used in this type of work should be made of an alkali-free resistant glass, *e.g.*, pyrex.

Preparation of Cultures.—Approximately 1 gm. of minced chick embryo tissue to each 5 cc. of Tyrode's solution was used. Proper amounts of tissue suspended in its vehicle were distributed in flasks. The medium was then inoculated with 0.25 cc. of virus emulsion, the mouth of each flask containing its cotton plug was securely covered with several layers of tin-foil, and the cultures were incubated at 37°C. for 5 days. New cultures were made by direct transfer of 0.25 cc. of an old culture into flasks of fresh medium. In this simple manner, vaccine virus can be propagated through an indefinite number of culture generations. One should be careful not to use too much tissue for a given amount of Tyrode's solution, inasmuch as it appears that an optimum ratio between tissue and fluid exists for the maximal multiplication of virus.

Tests for Sterility of the Cultures.—Early in the work, the medium was tested for sterility before it was inoculated with virus. During the interval (48 hours), the prepared flasks were stored at +5°C. Later these preliminary tests were omitted to advantage. Two separate batches of media were prepared for each

transfer in order to escape the possibility of having the work delayed by bacterial contaminations. After the cultures had been incubated for 3 days, a small amount of material was removed from each one and seeded in broth tubes and on blood agar plates. After the virus cultures had been incubated for 5 days, smears from each one were prepared, stained, and examined for bacteria. The material in the flasks that was found by cultural tests and smears to be free from bacteria was used for transfers and animal inoculations. Numerous flasks have been handled in the manner described above and only 1 in 12 became contaminated with bacteria.

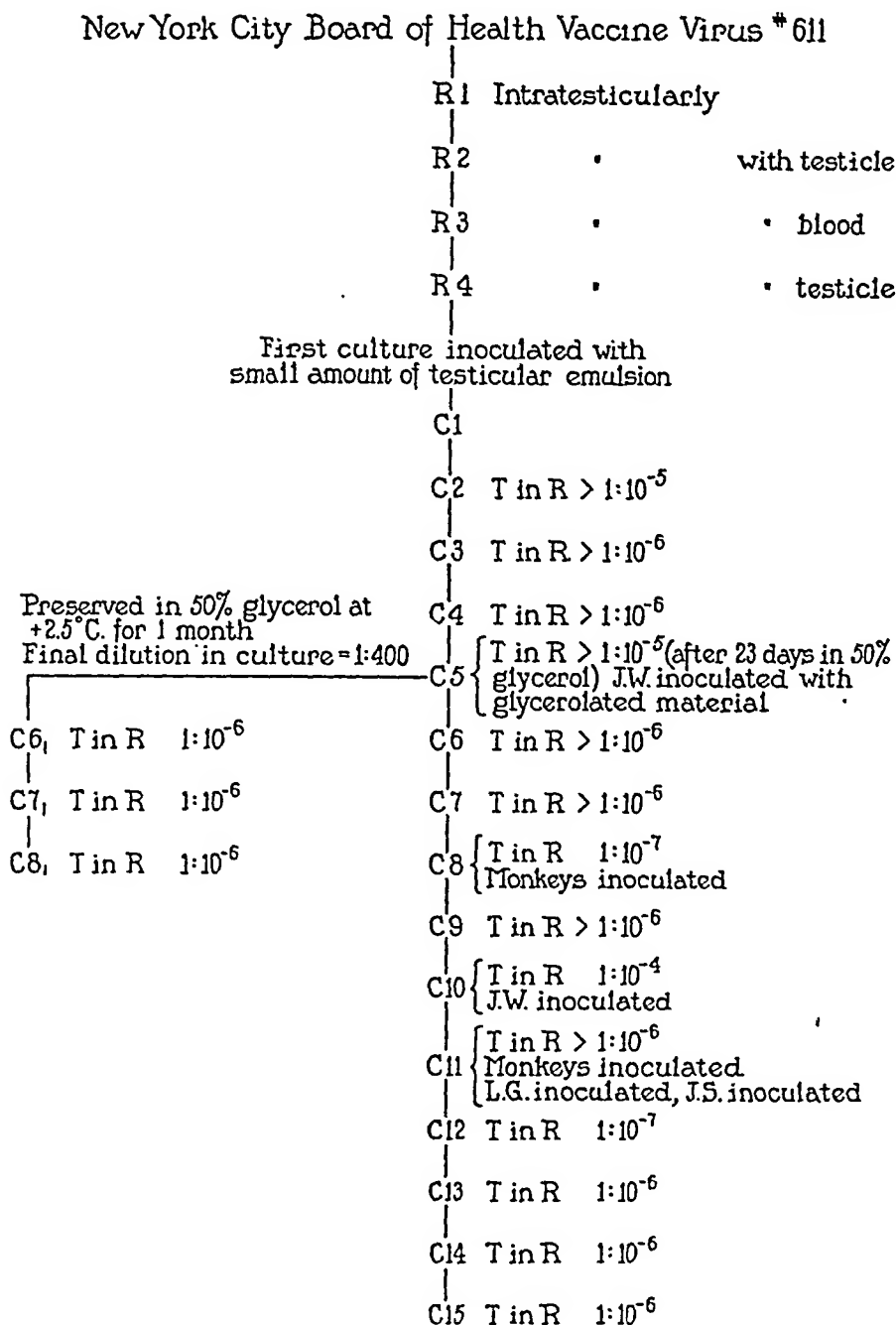
Tests for Potency of Virus.—Cultures for titration were ground in sterile mortars, and then appropriate dilutions were made with Locke's solution. A fresh pipette was always used for each successive dilution. 0.25 cc. of each dilution were injected into the shaved skin or were smeared on the scarified shaved skin of rabbits. In addition to rabbits, monkeys and human volunteers were employed for the potency tests of the virus.

Preservation of the Virus.—Many workers have stated that culture virus is difficult to preserve. This is true of viruses cultivated in certain kinds of media. It must be borne in mind, however, that no vaccine virus keeps indefinitely above 0°C. Furthermore, when glycerol is used, care must be exercised in selecting one that will not inactivate the virus. Merck's (P-W-R Analytical Chemicals) glycerol has been found suitable for this work.

EXPERIMENTAL

The vaccine virus distributed by the New York City Board of Health is prepared on calves, ripened in glycerol at about $-8^{\circ}\text{C}.$, and then treated with brilliant green. The bacterial count in such material is low. Indeed, at times, the virus seems to be entirely free from viable microorganisms. A specimen from Lot 611 was obtained from the Board of Health Laboratories, and with it cultures were initiated. At first it appeared that we had been fortunate enough to obtain a virus free from bacteria, but soon it was discovered that a very slow-growing diphtheroid was present as a contaminant. Consequently we were faced with the problem of how to rid the virus cultures of the bacteria. Filtration was first considered as a means of accomplishing this, but it was not attempted because vaccine virus filters with difficulty. Then we decided to try passage through rabbits in the following manner.

Rabbit 1 received in each testicle 1 cc. of a culture containing diphtheroids and vaccine virus. 4 days later the testicles were removed and emulsified with Locke's solution. 1 cc. of the emulsion was injected into each testicle of Rabbit 2. After



TEXT-FIG. 1. Summary of experiments in which a dermal strain of vaccine virus was adapted to a culture medium consisting of minced chick embryo suspended in Tyrode's solution. T in R = titer in rabbit.

a lapse of 4 days, blood (4) was removed¹ aseptically from the heart of Rabbit 2 and immediately injected in 1 cc. amounts into the testicles of Rabbit 3. This animal developed fever on the 4th day, and small hard nodules were felt in the testicles. At this time the testicles were removed and emulsified with Locke's solution. 1 cc. of the emulsion was inoculated in each testicle of Rabbit 4. After 4 days these testicles were removed and ground in a mortar without sand. Approximately 20 cc. of Tyrode's solution were thoroughly mixed with the macerated tissue. The emulsion was tested for the presence of bacteria and was found to be sterile. Then small amounts (0.25 cc.) of the supernatant material from a centrifuged specimen of the emulsion were used to initiate a series of virus cultures (see Text-fig. 1).

Having rid the dermal vaccine virus of bacteria it remained for us to determine whether it was possible to adapt such a strain to our method of cultivation. Proceeding in the manner described above, we have been able to carry the virus through an uninterrupted series of 15 culture generations (Text-fig. 1), and there is no reason to suppose that it cannot be cultivated indefinitely. The records of the titrations of the virus shown in Text-fig. 1 leave no room for doubt concerning the multiplication of the active agent. Furthermore, fresh cultures have been initiated with glycerolated material (Text-fig. 1) stored for 30 days at $+2.5^{\circ}\text{C}$. Such facts make it evident that vaccine virus is capable of indefinite propagation in cultures without the intervention of animal passage.

Reactions in Animals

It has been shown that a dermal strain of vaccine virus is capable of multiplication in a medium consisting of minced chick embryo suspended in Tyrode's solution. The question as to whether the virus has been altered by such treatment naturally arises. To investigate this matter, rabbits and monkeys were employed.

Material from each set of cultures, with the exception of the 1st, has been tested by intradermal inoculations in the shaved skin of rabbits. The reactions caused by the virus have not been altered through the process of cultivation, and have always been similar to those produced by a potent strain of vaccine virus unassociated with bacteria. This fact is illustrated by Fig. 1 that portrays lesions following intradermal injections of different dilutions of a culture in the 14th generation. In addition to intradermal injections, inoculations on the scarified shaved skin of

¹ All operations were performed under ether anesthesia.

rabbits have been made from time to time. Following such inoculations, typical vaccinal lesions unassociated with hemorrhage and necrosis developed. Fig. 2 represents lesions produced by different dilutions of a culture in the 6th generation. Finally, it has been shown that the culture virus smeared on the scarified skin of monkeys (*Macacus rhesus*) induces typical vaccinal lesions that heal rapidly without resultant injury to the animals.

From the above experiments it is obvious that the culture virus caused typical vaccinal lesions in animals. We then became interested in determining whether the active agent had undergone immunological changes. Consequently the following cross immunity experiments were performed.

Rabbits inoculated respectively with virus from the 6th, 8th, 10th, and 11th culture generations were allowed to recover. Then, they and 2 normal stock rabbits were inoculated with New York City Board of Health vaccine virus, Lot 617. The recovered animals were refractory while the normal ones developed typical vaccinal lesions.

The above experiment indicates that repeated cultivation of the virus in a medium consisting of minced chick embryo suspended in Tyrode's solution did not alter its antigenic properties.

Reactions in Human Volunteers

Not infrequently we receive requests for vaccination against small-pox. Since there was no valid reason for not using the culture virus in human beings, we decided, with the consent of the patients or of their parents, to substitute in certain instances the culture vaccine for calf lymph. The results obtained in 3 children are detailed below.

J. W., aged 2 years, May 2, was vaccinated at 2 points on the outer surface of the upper left arm. The superior inoculation was made with material from a 5th-generation culture that had been preserved in 50 per cent glycerol at $+2.5^{\circ}\text{C}$. for 28 days. The inferior inoculation was made with material from a 10th-generation culture that had been preserved in 50 per cent glycerol at $+2.5^{\circ}\text{C}$. for 2 days. The method of vaccination consisted of a small amount of the virus being smeared over an area of skin previously superficially scarified. May 3, superficial marks at points of inoculation. May 4, no change. May 5, 9:00 a.m., both lesions, the lower slightly more than the upper, show some swelling and elevation. 2:30 p.m., there have appeared, particularly over the area of the lower inoculation, crops of small vesicles (Fig. 3). No fever. May 6, lesions well developed. Both are small, the upper being about 0.5 cm. in diameter, the lower about 1.0 cm. (Fig.

4). Temperature rose to 101.2°F. *May 7*, lesions have increased in diameter (Fig. 5). Temperature rose to 101.6°F. *May 8*, lesions are still increasing in size and are now pustular (Fig. 6). Temperature rose to 102°F. Further development of the lesions not observed, because the child had to leave town with its parents.

L. G., aged 22 months, *May 11*, was vaccinated at one point on the outer surface of upper left arm with material from an 11th-generation culture that had been preserved in 50 per cent glycerol at +2.5°C. for 11 days. *May 12*, no evidence of inflammation. *May 13*, vaccinated area red but not elevated (Fig. 11). *May 14*, at point of inoculation, there is a red papule (Fig. 12). *May 15*, several small vesicles have appeared (Fig. 13). *May 16*, vesicles have coalesced and are beginning to be pustular. Narrow primary areola is present (Fig. 14). *May 17*, lesion somewhat larger, no induration, no fever. *May 18*, lesion has increased in size and is beginning to dry in center (Fig. 15); no fever. *May 19*, pustule larger (Fig. 16), no induration, no fever. *May 20*, pustule larger, drying; secondary areola 3 mm. in width; slight amount of induration (Fig. 17). Temperature rose to 100.4°F. *May 21*, pustule drying, secondary areola 4 mm. in width, induration more marked (Fig. 18). Temperature 100.0°F. *May 22*, secondary areola 1 cm. in diameter, induration more marked (Fig. 19); no fever. Patient was reinoculated on right arm with New York City Board of Health vaccine virus, Lot 617. *May 23*, primary vaccination: secondary areola has almost disappeared, induration less (Fig. 20). Secondary vaccination: skin at point of inoculation red. No fever. *May 25*, primary vaccination: lesion covered by dry scab; secondary areola and induration no longer present (Fig. 21). Secondary vaccination: slightly red. *May 26*, primary vaccination: only a dry scab remains. Secondary vaccination: negative. *May 28*, primary vaccination: the scab will drop off in a few days (Fig. 22). Secondary vaccination: negative. Patient in excellent condition and discharged from the hospital. The virus with which this patient was reinoculated was shown to be potent by a successful vaccination of a normal person. Thus, the lack of response to the secondary inoculation indicates that a state of immunity had been induced by the primary vaccination.

After it had been demonstrated that the culture virus produces without danger a typical vaccinia in man and that it induces an immunity against calf lymph, we decided, in view of the fact that the culture vaccine seemed to possess a greater potency than does ordinary calf lymph, to compare the action of the two kinds of active agent.

M. S., aged 11 months, and J. S., aged 22 months, were vaccinated; the former with New York City Board of Health virus, Lot 617, that had been stored in 50 per cent glycerol below 0°C. for more than a year, the latter with virus from an 11th-generation culture that had been preserved in 50 per cent glycerol at +2.5°C. for 21 days. Typical vaccinia developed in both children who showed no evi-

dences of illness except a local reaction and a slight elevation of temperature, 101.5°F. Certain differences, however, were noted in the course of the infection in the 2 patients, *viz.*, the child who was inoculated with the culture virus had a more prolonged febrile reaction and developed more redness and induration associated with a few secondary vesicles around the vaccinal pustule than did the infant who received the calf lymph (Figs. 7 to 10).

The experiment just described and others that will not be presented at this time indicate that the culture virus is very potent. The fact that fresh culture vaccine produces considerable reaction is not surprising, inasmuch as it has long been known that a "green" virus is more likely to give rise to redness, induration, and secondary vesicles than is one properly aged. Therefore, the culture virus should be allowed to ripen before it is used for vaccination of human beings.

DISCUSSION

The medium employed for the *in vitro* cultivation of vaccine virus is exceedingly simple, but one must remember that it contains living cells. In spite of recent reports (5) to the contrary, no one has definitely shown (6, 7) that vaccine virus is capable of pullulation in the absence of surviving susceptible cells.

Several kinds of media have been devised for the production of vaccine virus. The one described in this paper, however, is the best of the lot already proposed, because, inasmuch as neither serum nor plasma is used and since the tissue consists of minced chick embryo, it is the least likely to become contaminated by an extraneous virus injurious to man. Furthermore, the virus produced in this manner remains potent in 50 per cent glycerol at +2.5°C. for at least a month. More information, however, concerning the length of time the culture vaccine remains active under a variety of conditions should be obtained and to this end experiments are now being conducted.

SUMMARY

1. A dermal strain of vaccine virus has been adapted to a simple culture medium consisting of minced chick embryo suspended in Tyrode's solution.
2. The bacteria-free culture virus, thus obtained, produces in lower animals and in man typical vaccinia that renders them refractory to infection with ordinary vaccine virus harvested from calves.

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EXPLANATION OF PLATES

PLATE 45

FIG. 1. Lesions produced in a rabbit by intradermal inoculations (0.25 cc.) of 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} dilutions, respectively, of a 14th-generation culture of vaccine virus. $\times 1$.

FIG. 2. Lesions induced in a rabbit by different dilutions (undiluted, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}) of a 6th-generation culture of vaccine virus. $\times 1$.

PLATE 46

FIGS. 3 to 6. 3rd-, 4th-, 5th-, and 6th-day vaccinal lesions, respectively, produced in J. W. by culture virus: the upper resulted from an inoculation of a 5th-generation culture, the lower from an infection with a 10th-generation culture. $\times 1$.

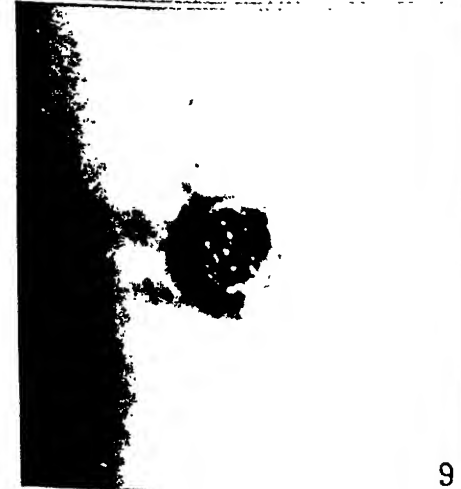
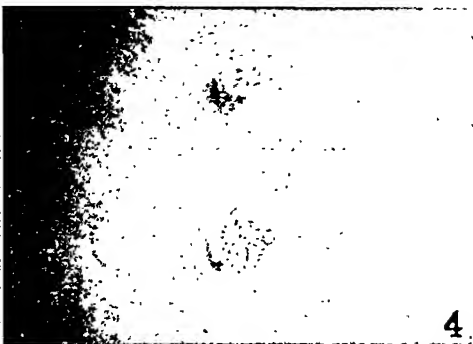
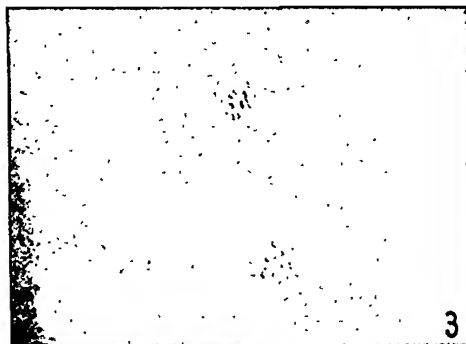
FIGS. 7, 9. 6th- and 9th-day vaccinal lesions, respectively, produced in M. S. by New York City Board of Health virus, Lot 617. Compare with Figs. 8 and 10. $\times 1$.

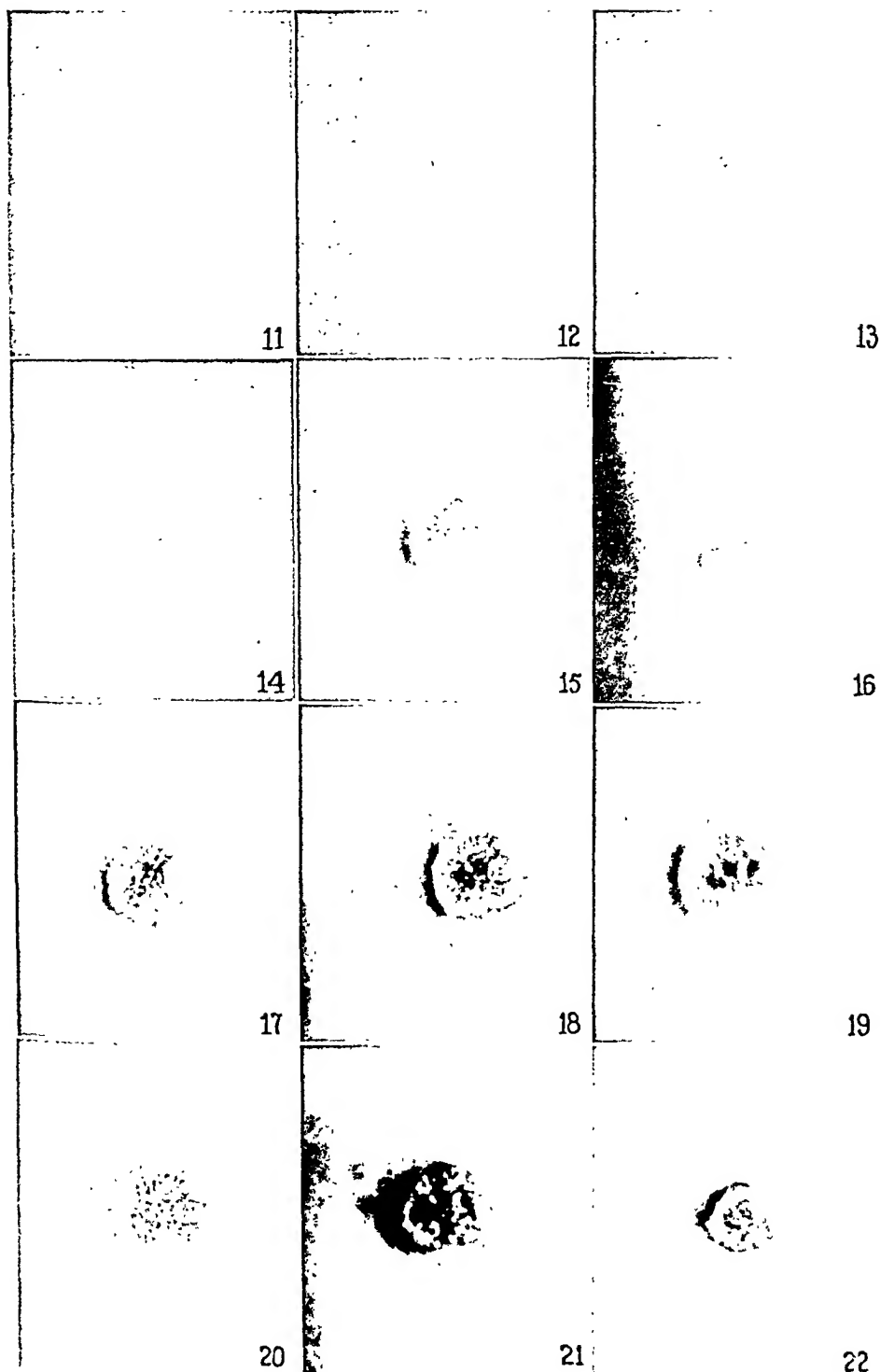
FIGS. 8, 10. 6th- and 9th-day vaccinal lesions, respectively, induced in J. S. by an 11th-generation culture. Comparison of these reactions with those in Figs. 7 and 9 shows that the culture vaccine, only 21 days old, caused more redness and secondary vesicles than did the City Board of Health virus that had been aged for more than a year. $\times 1$.

PLATE 47

FIGS. 11 to 22. 2nd-, 3rd-, 4th-, 5th-, 7th-, 8th-, 9th-, 10th-, 11th-, 12th-, 13th-, and 18th-day vaccinal lesions, respectively, produced in L. G. by a 10th-generation culture. The photographs portray the complete evolution of a vaccinal lesion induced in man by a dermal strain of virus adapted to *in vitro* cultivation. $\times 1$.







SPECIFICITY OF THE PRECIPITIN REACTION IN TOBACCO MOSAIC DISEASE*

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The results of previous investigations by the writer (Purdy, 1928, 1929) have shown that when rabbits receive a series of injections of saline extracts of leaves from tobacco plants affected with the filterable virus of common field mosaic disease, the serum of these animals forms a heavy precipitate when mixed with virus extract and incubated for a suitable period. No precipitate occurs, however, when the virus extract is added to serum drawn from a rabbit prior to injection of the plant juice. It has been shown further that precipitins for leaf extracts of normal tobacco plants can also be produced in rabbits by the same method of hyperimmunization. Serum from animals injected with juice from normal tobacco leaves forms a precipitate upon the addition of extracts of either normal or diseased tobacco; antiserum for virus extract likewise contains precipitins for juice from normal as well as from diseased tobacco leaves. However, the reaction differs noticeably in the amount of precipitate formed in the various combinations of the two antisera and plant extracts. The precipitate resulting from a mixture of juice from normal tobacco and either antiserum is never heavy and rarely occurs in a saline dilution of antiserum exceeding 1:9. Likewise, in mixtures of virus extract and antiserum for juice from normal tobacco leaves, only a slight precipitation occurs and only in low dilutions of antiserum. The largest amount of precipitate is produced in a mixture of virus extract and antiserum for virus extract, the homologous antiserum. Although in the preparation of the plant extracts the same quantity of normal and diseased leaves by green weight may be used in each case for a given volume of saline solution, antiserum for virus extract of high precipitin titer will react with normal juice to form a precipitate in a dilution of antiserum, rarely exceeding 1:9. Upon the addition of virus extract to virus antiserum, a precipitate may form in a dilution as high as 1:2000. The observation that virus extract reacts more readily with the homologous antiserum led to the assumption that there might be some substance present in virus extract not found in normal tobacco leaf extract that is responsible for the reaction. Con-

* These investigations were begun during the tenure of a National Research Council Fellowship in the Biological Sciences.

sequently, precipitin-absorption experiments were undertaken in which the precipitins for normal tobacco extract were absorbed from the antiserum for virus extract by adding small amounts of juice from normal tobacco leaves and incubating the mixtures for $1\frac{1}{2}$ hours at 37°C . After standing overnight in the ice chest, the mixtures were centrifugalized to throw down the precipitate. The supernatant fluid was then drawn off and more normal juice was added to the partially absorbed serum. The process was continued until no precipitate was formed upon the addition of normal juice, whereupon the absorption was regarded as complete. The amount of normal juice necessary for complete absorption of the precipitins was small, so that the dilution of the antiserum by the addition of normal juice is a negligible factor. The subsequent addition of virus extract to the antiserum from which all of the precipitins for normal juice had been absorbed resulted in the formation of a heavy flocculent precipitate. These results were interpreted as evidence of the presence of material in virus extract not found in normal juice and antigenic in nature, *i.e.* capable of stimulating the production of antibodies, in this instance precipitins, upon injection into an animal.

It was suggested that the antigenic material specific for virus extract and not present in extracts of normal tobacco leaves might be either foreign protein or protein of the tobacco plant altered by mosaic disease, with a consequent change in its antigenic property. If foreign protein, two possibilities are open to consideration, the antigenic material might be virus itself or protein from microorganisms present in the mosaic diseased plant in the rôle of secondary invaders.

Matsumoto (1930) using a similar method of hyperimmunization likewise produced precipitins in rabbits for virus extracts of tobacco affected with mosaic disease.

In earlier experiments, it was also shown by the present author that leaf extracts of tomato, pepper, and petunia, affected with the virus of common field mosaic disease, react with antiserum for virus extract of tobacco to form specific precipitates. Matsumoto and Somazawa (1930) hyperimmunized rabbits separately to virus extracts of both tobacco and tomato. They then demonstrated by precipitin-absorption experiments that all precipitins in either antiserum could be removed by complete absorption with the virus extract of either tobacco or tomato, thus proving the identity of the specific antigenic material in the case of both hosts of the virus.

It was thought that more evidence concerning the nature of the antigenic material specific for virus extract might be obtained by a further investigation of the specificity of the precipitin reaction. Three main lines of attack were planned. Only one kind of antiserum

was used throughout the experiments, namely the antiserum for virus extract of tobacco (*Nicotiana tabacum* L. var. Turkish) affected with common field mosaic disease. However, the source of virus extracts employed in the precipitin tests was varied. In one set of experiments, leaf extracts were prepared from other plants than tobacco, affected with virus diseases distinct from tobacco mosaic. This source of virus extract was used on the assumption that if the specific antigenic material in tobacco virus extract were altered tobacco protein, similar substances might conceivably be found in different virus diseases as a result of a like alteration of normal host protein.

In another set of tests, leaf extracts of different hosts affected with the same virus, that of the common field mosaic of tobacco, were tested for the presence of material reacting with the specific precipitins in antiserum for tobacco virus extract. It was thought desirable to determine whether or not all hosts of the virus available for tests would give a positive precipitin reaction with the antiserum.

Finally, a set of experiments was undertaken in which two other viruses, distinct from that of tobacco mosaic, were multiplied separately in a common host, Turkish tobacco, which is also a host of tobacco mosaic virus. Leaf extracts of the common host affected with these different viruses were tested with the antiserum for tobacco mosaic virus extract for substances capable of reacting with the specific precipitins. By such a procedure it seemed likely that some evidence might be obtained relative to the nature of the antigenic material present in virus extract and not found in normal tobacco leaves.

EXPERIMENTAL PROCEDURE

Preparation of Antigens.—All plant extracts were prepared by grinding green leaves to a pulp in a mortar with saline (0.85 per cent) solution. The macerated tissue was then removed by passage through coarse filter paper. A clear filtrate was then obtained by using a combination of Nos. 5 and 42 hard filter papers (Whatman).

Hyperimmunization of Rabbits.—The method described in detail in an earlier article (Purdy, 1929) was followed in general, except that in addition to the intravenous route for injection, the intraperitoneal was employed with equally good results. It was also found that after a rest period of about 20 days following the first five injections and then after every two injections for a couple of times, the

precipitin titer of the antiserum was markedly increased.¹ When the intravenous route was employed for hyperimmunization, only 0.5 cc. of antigen was administered at the first injection immediately following a rest period in order to avoid possible anaphylactic shock. The remainder of the antigen, usually about 1 cc., was injected an hour later.

Precipitin Experiments.—0.3 cc. each of undiluted antigen and antiserum, undiluted or in a dilution of 1:4, were thoroughly mixed and the contents of the tubes were incubated for 1½ hours in a water bath at 37°C. Upon removal from the water bath the tubes were examined for the presence of precipitate and placed in the ice chest overnight. The following morning a final record was made of the presence or absence of precipitate. The precipitin tests were repeated several times with each antigen employed. In every set of tests control tubes were included, some containing antigen with saline solution in place of antiserum, others having antiserum with saline solution substituted for antigen.

Precipitin Tests with Extracts of Different Viruses of Plants Other than Tobacco

Sudan grass affected with the virus of sugar-cane mosaic, and *Hippeasium equestre* Herb. and lily affected with their respective mosaic diseases, were selected as representative of virus diseases with which intracellular bodies are associated resembling those found in tobacco mosaic disease. In the case of these four maladies not only the macroscopic but the microscopic symptoms of the diseases bear a similarity to one another. Mosaic diseased plants of *Abutilon striatum* Dicks. (*A. Thompsoni* hort.) were chosen as typical of infectious chloroses transmissible by grafting, while yellows of peach was taken to represent another type of infectious chlorosis distinct from the mosaic type, but with symptoms sufficiently characteristic to include the disease in the class attributable by Kunkel (1928) and others to filterable viruses. In none of the maladies referred to, except tobacco mosaic, has actual filtration of the virus been demonstrated, but as Kunkel points out they may be regarded as members of the group of diseases caused by viruses inasmuch as they produce a set of symptoms in their hosts characteristic of known filterable viruses and distinct from the symptoms resulting from infections caused by recognized plant parasites such as bacteria and fungi. In the case of many diseases included in the group due to filterable viruses, lack of

¹ This observation was made during a series of experiments carried out in collaboration with Mr. Joseph Webb.

ability to transmit the diseases mechanically has rendered proof of the filterability of the infectious agents impossible.

Extracts of these various plants, Sudan grass, *Hippeastrum*, lily, *Abutilon*, and peach, affected with different viruses were mixed with the antiserum for virus extract of tobacco affected with mosaic disease.

TABLE I

Precipitin Tests with Antiserum for Tobacco Mosaic Virus and Extracts of Various Plants Affected with Different Viruses

Antiserum	Antigen		Precipitate*
	Host	Disease	
Undiluted	Sudan grass	Mosaic	0
(1:4) dil.	" "	"	0
Undiluted	<i>Hippeastrum equestre</i>	"	0
(1:4) dil.	" "	"	0
Undiluted	Lily	"	0
(1:4) dil.	"	"	0
Undiluted	<i>Abutilon striatum</i>	"	0
(1:4) dil.	(<i>A. Thompsoni</i> hort.) " "	"	0
Undiluted	Peach	Yellows	0
(1:4) dil.	"	"	0
Undiluted	<i>Nicotiana tabacum</i>	Tobacco mosaic	++++
(1:4) dil.	var. Turkish " "	" "	++++

* 0 = no precipitate.

± = very slight precipitate.

+ = slight precipitate.

++ = moderate precipitate.

+++ = heavy precipitate.

++++ = very heavy precipitate.

After an incubation period of 1½ hours the tubes were placed in the ice chest and on the following morning a record was made of the presence or absence of precipitate. The antiserum was used undiluted and in a dilution of 1:4. Tubes containing a mixture of antiserum and the homologous antigen were included in each set of experiments.

Results.—Extracts of *Hippeastrum equestre*, lily, and *Abutilon striatum* (*A. Thompsoni* hort.), affected with the viruses of their respective mosaic diseases; Sudan grass, affected with the virus of sugar-cane mosaic; and peach, affected with yellows, formed no precipitate with antiserum for virus extract of tobacco mosaic disease (see Table I).

Precipitin Tests with Different Plant Extracts of Tobacco Mosaic Virus

It has been demonstrated previously (Purdy, 1929) that extracts of tomato, pepper, and petunia affected with the virus of tobacco mosaic disease form heavy precipitates when added to the antiserum for virus extract from tobacco leaves. Matsumoto and Somazawa (1930) have further shown that the precipitins in antiserum for the virus extract of tobacco and tomato are apparently identical. Other plants reported here have been added to the list of hosts of tobacco mosaic virus already tested for the ability to precipitate antiserum for virus extract of tobacco. Saline extracts of *Nicotiana glutinosa* L. and *N. rustica* L. affected with the virus were tested with the antiserum. All the hosts of tobacco mosaic virus so far reported as sources of virus in the precipitin tests are members of the Solanaceae and only a few hosts outside of this family are mentioned in the literature, to which the virus has been successfully transmitted. Among them are bean (Price, 1930), the crookneck squash (Elmer, 1922), and *Martynia louisiana* Mill. (Elmer, 1925). Bean and squash have not yet been tested but *Martynia*, infected with the virus of tobacco mosaic, has been used as a source of virus extract to determine its ability to form a precipitate when mixed with equal parts of the antiserum. *Martynia* belongs to the family Martyniaceae, which is closely related to the Solanaceae.

Results.—Leaf extracts of *Nicotiana rustica*, *N. glutinosa*, and *Martynia* affected with tobacco mosaic virus precipitated the antiserum for the same virus obtained from Turkish tobacco (see Table II).

Precipitin Tests with Tobacco Extracts of Three Distinct Viruses

Two virus diseases of Turkish tobacco distinct from tobacco mosaic were selected to test further the specificity of the precipitin reaction for a given strain of virus on the same host plant that was used for

hyperimmunization. Ringspot disease and cucumber mosaic are both successfully reproduced in Turkish tobacco. On the basis commonly used at present for differentiation of the plant viruses, these can be identified as distinct from the virus causing tobacco mosaic

TABLE II

Precipitin Tests with Antiserum for Tobacco Mosaic Virus and Extracts of Tobacco Mosaic Virus on Various Hosts

Antiserum	Antigen		Precipitate*
	Host	Disease	
Undiluted (1:4) dil.	Tomato "	Tobacco mosaic " "	++++ ++++
Undiluted (1:4) dil.	Pepper "	" "	++++ ++++
Undiluted (1:4) dil.	Petunia "	" "	++++ ++++
Undiluted (1:4) dil.	<i>Nicotiana rustica</i> " "	" "	++++ ++++
Undiluted (1:4) dil.	<i>Nicotiana glutinosa</i> " "	" "	++ +
Undiluted (1:4) dil.	<i>Martynia louisiana</i> " "	" "	++++ ++++
Undiluted (1:4) dil.	<i>Nicotiana tabacum</i> var. Turkish " "	" "	++++ ++++

* 0 = no precipitate.

± = very slight precipitate.

+ = slight precipitate.

++ = moderate precipitate.

+++ = heavy precipitate.

++++ = very heavy precipitate.

disease. The distinguishing features, recently enumerated by Johnson and Hoggan (1931), are to be found in the symptom expression, the properties of virus extracts, the modes of transmission of the viruses, and the cytological picture.

Extracts of ringspot, cucumber, and tobacco mosaic viruses multiplied separately in the common host, Turkish tobacco, were mixed with the antiserum to determine the presence or absence of substances reacting with the specific precipitins.

Another source of cucumber mosaic virus was utilized in these tests. *Nicotiana glutinosa* was employed for this purpose since it is a host

TABLE III

Precipitin Tests with Antiserum for Tobacco Mosaic Virus and Extracts of Tobacco Affected with Three Distinct Viruses

Antiserum	Antigen		Precipitate*
	Host	Disease	
Undiluted	<i>Nicotiana tabacum</i>	Ringspot	±
(1:4) dil.	“ “	“	0
Undiluted	“ “	Cucumber mosaic	±
(1:4) dil.	“ “	“ “	0
Undiluted	<i>Nicotiana glutinosa</i>	“ “	±
(1:4) dil.	“ “	“ “	0
Undiluted	<i>Nicotiana tabacum</i>	Tobacco “	++++
(1:4) dil.	“ “	“ “	++++

*0 = no precipitate.

± = very slight precipitate.

+ = slight precipitate.

++ = moderate precipitate.

+++ = heavy precipitate.

++++ = very heavy precipitate.

common to the viruses of both cucumber and tobacco mosaic diseases. While the tobacco mosaic virus remains localized in this plant, the cucumber mosaic virus produces a systemic infection in this host. Extracts of *N. glutinosa* affected with cucumber mosaic virus were tested with the antiserum for tobacco mosaic virus extract.

Results.—Leaf extracts of Turkish tobacco affected with ringspot or cucumber mosaic disease gave only a slight precipitate with undiluted

antiserum for tobacco mosaic virus on the same host and no precipitate with antiserum in a dilution of 1:4. Juice from *Nicotiana glutinosa* affected with cucumber mosaic virus produced only a slight precipitate with undiluted antiserum and no precipitate with antiserum in a dilution of 1:4 (see Table III).

A heavy flocculent precipitate formed in every tube containing tobacco mosaic virus extract from Turkish tobacco and the homologous antiserum (see Tables I, II, and III).

No precipitation occurred in any of the control tubes of antigen or antiserum.

DISCUSSION

By reference to Table III it will be noted that a slight precipitation occurs in mixtures of undiluted antiserum and every antigen prepared from tobacco. This is attributed to the presence of precipitins for normal tobacco juice. Precipitation occurs when leaf extracts of healthy Turkish tobacco, tomato, petunia, and pepper are mixed with antiserum for either normal tobacco extract or tobacco virus extract. The reaction occurs only in undiluted antiserum or in dilutions usually not exceeding 1:4. When a clear filtrate of a concentrated extract of normal tobacco leaves is added to a 1:4 or a 1:9 dilution of an antiserum for tobacco virus having a high precipitin titer, a definite precipitate forms, while the same antigen added to normal rabbit serum gives an entirely negative reaction. Also virus extracts of Turkish tobacco react with low dilutions of antiserum for normal tobacco juice to produce a slight precipitate, while normal rabbit serum fails to react. Therefore, there seems to be little doubt that when antigens are prepared from hosts identical with the one used for the purpose of hyperimmunization or from closely related hosts, a small part of the precipitate formed in mixtures of antiserum is due to precipitins for normal tobacco juice. The reaction can be readily eliminated by dilution of the antiserum and the use of a less concentrated antigen.

There has been no attempt to prove from the evidence presented in this paper that the viruses of cucumber mosaic and ringspot diseases may not be related serologically to the tobacco mosaic virus. Since a positive precipitin reaction is obtained only in mixtures of undiluted antiserum and antigen, any dilution of the antiserum, necessary for

obtaining proof by the use of precipitin-absorption tests, would be objectionable. Evidence of a possible relationship of these three viruses would seem to depend upon precipitin tests between the antiserum for virus extract of tobacco mosaic and extracts prepared from hosts of the ringspot and cucumber viruses unrelated to tobacco.

Owing to the possibility of the existence of a prozone phenomenon in the precipitin reactions, the antigens and antiserum were tested in various dilutions but only with negative results when the antiserum dilution exceeded 1:4, except in the case of the antigens from plants affected with the virus of tobacco mosaic disease. When undiluted antiserum of a high precipitin titer is used with the homologous antigen, the formation of a precipitate is frequently delayed although a flocculation eventually takes place.

As shown in Table II, the reaction between the antiserum and virus extract of *Nicotiana glutinosa* is recorded as moderate or in the case of diluted antiserum, only slight. The virus produces localized lesions in this host and according to Holmes (1929) the virus concentration is never great. Larger quantities of leaves of *N. glutinosa* extracted with a given volume of saline solution but having a low virus content, precipitate antiserum less than smaller amounts of the leaves of other hosts of the virus having a higher virus content.

Since microorganisms are frequently associated with mosaic-diseased plants, the possibility that the specific precipitate is due to such foreign protein should not be ignored. If the reaction were attributable to such concomitant antigens, the results would indicate that these microorganisms are present in every host of the virus thus far tested and are found in tobacco affected with the common field mosaic virus, and not in tobacco affected with the viruses of either cucumber mosaic or ringspot disease.

SUMMARY AND CONCLUSIONS

1. Leaf extracts of Sudan grass, *Hippocrastrum equestre* Herb., lily, and *Abutilon striatum* Dicks. (*A. Thompsoni* hort.), each affected with its respective mosaic disease, and peach affected with yellows disease, were tested for their ability to precipitate antiserum for virus extract of tobacco mosaic disease. No precipitate occurred.

2. *Nicotiana glutinosa* L., *N. rustica* L., and *Martynia louisiana* Mill.

were added to the list of hosts of tobacco mosaic virus which have been tested with antiserum for the same virus in *N. tabacum* L. var. Turkish. The object was to determine the presence or absence of material reacting with the specific precipitins such as that already demonstrated in extracts of tomato, pepper, and petunia affected with the same virus. The presence of specific substances was demonstrated in every case.

3. The viruses of ringspot and cucumber mosaic diseases were multiplied in Turkish tobacco and leaf extracts of the affected plants were used in turn as antigens in precipitin tests with antiserum for tobacco mosaic virus extract of Turkish tobacco. A slight precipitation resulted in the tubes containing undiluted antiserum and virus extract such as occurs when juice from normal tobacco is used with undiluted antiserum. No precipitate was demonstrable that was specific for virus extracts of tobacco affected with either ringspot or cucumber mosaic disease.

4. The results favor the interpretation that the specific antigenic substance in virus extract of tobacco mosaic disease is foreign antigenic material, possibly virus itself, not altered host protein.

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STUDIES ON THE BLOOD CYTOLOGY OF THE RABBIT

VIII. THE BLOOD OF NORMAL RABBITS AS AN INDEX OF THEIR RESISTANCE TO A TRANSPLANTABLE NEOPLASM

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This paper reports an investigation in connection with the study of animal constitution as a factor in susceptibility and resistance to disease (1). Blood was the constitutional factor studied because of the basis for work furnished by preceding papers which deal with the distribution, the spontaneous variations, and the interrelationships of the blood cells in the normal rabbit (2-7). A malignant neoplasm was chosen as the disease to be followed because of the knowledge already acquired concerning the blood reaction in rabbits inoculated with the tumor (8).

Two fundamental considerations underlie the present statistical investigation of the relationship between the blood cytology of normal rabbits and their susceptibility to this malignant disease. First, the malignancy of the transplantable neoplasm as observed in this laboratory varies from season to season and year to year, both as concerns groups of animals and among the individuals of the groups (9-11). Second, the cellular elements of the blood of normal rabbits vary seasonally and yearly, and appreciable differences in the cell formula occur both between groups (3-7) and individuals (2).

Material and Methods

Data have been obtained from 2 groups of rabbits inoculated with the Brown and Pearce rabbit tumor. The first group comprised 78 rabbits which were the same as those used in the experiments on which the blood reaction to tumor inoculation was reported (8). A description of the animals and of the experimental methods employed has already been given. The animals which were used for working out the methods of correlation were distributed as follows:

TABLE I

Group	No. of rabbits	First blood examination	Last blood examination	Tumor inoculation	No. of blood examinations before inoculation*	No. of weeks in laboratory before inoculation
1	10†	Oct. 24, 1927	Nov. 16, 1927	Nov. 17, 1927	4	4½
2	5	Dec. 5, 1927	Jan. 5, 1928	Jan. 5, 1928	12	4½
3	9	Feb. 8, 1928	Feb. 23, 1928	Feb. 24, 1928	4	2
4	10†	Apr. 6, 1928	Apr. 18, 1928	Apr. 20, 1928	4	2
5	10	Sept. 18, 1928	Nov. 22, 1928	Nov. 22, 1928	8	8
6	5†	Dec. 29, 1928	Jan. 3, 1929	Jan. 4, 1929	3	1
7	12	Oct. 26, 1929	Nov. 19, 1929	Nov. 19, 1929	7	4
8	12	Dec. 31, 1929	Jan. 14, 1930	Jan. 14, 1930	5	2
9	8	Dec. 31, 1929	Jan. 14, 1930	Jan. 14, 1930	5	26
Total...	78					

* In an earlier paper upon the blood cytology during the course of malignant disease (8), only those blood counts made within 4 weeks of inoculation were included. In the present paper all counts before inoculation are included without regard to the time factor.

† 1 rabbit each of Groups 1 and 4 was omitted because of a complicating nephritis; and an animal in Group 6 also because it was killed for transfer material before the end of the experiment.

A second series of 13 rabbits was used as a means of testing the accuracy of the methods developed with the first group; their distribution was as follows:

TABLE II

Group	No. of rabbits	Tumor inoculation	First blood examination	Last blood examination	No. of blood examinations before inoculation	No. of weeks in laboratory before inoculation
10	8	Sept. 17, 1930	Sept. 19, 1930	Sept. 19, 1930	2	2
11	5	Dec. 13, 1930	Dec. 13, 1930	Dec. 15, 1930	10	26
Total...	13				12	28

The hematological technique employed (2) included determinations of the total red and white cells with standardized pipettes, hemoglobin estimations by the Newcomer method, and neutral red supravital differential counts. 100 white cells were counted in each differential white cell determination. The number of blood counts made upon each group of animals before inoculation varied from 3 to

12 and they were distributed irregularly over the 1 to 10 weeks immediately preceding inoculation. The values used in this analysis are the *means of all counts* made upon each animal *before inoculation*.¹

The transplantable tumor is considered to be an epithelioma. It arose spontaneously in the scrotal skin of a rabbit 10 years ago (12) and has been carried from animal to animal by intratesticular inoculation for upwards of 100 generations.

Estimation of Malignancy.—Each experiment was terminated 2 months after inoculation and all surviving animals were killed by air embolism. A complete autopsy was made on every animal with especial reference to the distribution and the character of any tumor.

For the present statistical analysis, expressions of malignancy were reduced to a numerical basis by one of two methods. The first was the outcome of the experience that 2 months after inoculation, animals can be divided into four categories with respect to death or survival, namely: "deaths," "probable deaths," "probable recoveries," and "recoveries." Animals which at autopsy revealed no evidence of tumor, either at the site of inoculation or elsewhere are designated as "recoveries." Those which at autopsy showed only a slight tumor growth, usually in a necrotic condition, such as a residual primary lesion of the testicle, a circumscribed lymph node metastasis, or small peritoneal implantations, would presumably have recovered from the disease and, therefore, these individuals have been classed as "probable recoveries." The remaining animals which showed more or less widespread metastases of living tumor at autopsy have been classed as "probable deaths." In order to give a numerical value to the malignancy in each animal, the relative susceptibilities of the four classes of animals have been simply expressed as 4, 3, 2, and 1, respectively, or in percentage terms of malignancy as follows: "deaths" 100 per cent; "probable deaths" 67 per cent; "probable recoveries" 33 per cent; and "recoveries" 0 per cent.

This method of classification was used as the basis for studying the relation between the pre-inoculation blood findings and susceptibility or resistance to the tumor. The results obtained, however, were controlled by a second numerical method of appraisal. This method consists in determining the number of organs or tissues having tumor growth as determined by autopsy observation. Neither the number nor the extent of the growth in any organ nor the character of the growth is taken into consideration, but simply the presence or absence of a tumor focus. The number of possible foci has been arbitrarily set at 20, and the distribution of animal material upon this basis is as follows: "recoveries" 0 foci, "probable recoveries" from 1 to 4 foci, "probable deaths" from 4 to 12, and "deaths" from 4 to 20 foci. This second method of classification which gives a numerical value to the actual distribution of tumor in each animal has been included as

¹ The mean values used in the present analyses differ somewhat from those mentioned in the report on the blood reaction after inoculation, as has been stated in that paper (8).

supplementary evidence of the accuracy of the first method. In general, it has been found that the analytical results obtained with both methods are in substantial agreement.

Methods of Analysis and of Recording Results.—The method of determining the relation of the blood cells before inoculation to the course of the malignant disease has been to construct a simple correlation table for each type of cell studied. The four values for tumor malignancy have been placed along the ordinate and the values of the blood cell factor under consideration along the abscissa in an appropriate number of boxes. Simple averages of the malignancy of the tumor in those cases corresponding to each box along the abscissa were then made. Owing to the inadequate number of cases in some boxes, the box means were smoothed by the formula $\frac{S_A + 2S_B + S_C}{N_A + 2N_B + N_C}$ where S_B was the summation of the ordinate values in the box to be smoothed, S_A that of the preceding, and S_C that of the succeeding box; N_A , N_B , N_C represented the actual number of cases falling in the respective boxes. The smoothed box means were then connected by lines; the result of this procedure is shown in the curves of Text-figs. 1, 2, and 3. The small numbers beside each point in the curves represent the actual number of cases in each box before smoothing was done. The vertical line down the center of each chart represents the approximate modal value obtained upon normal rabbits in this laboratory.²

The values in the text-figures represent an analysis of the entire series of 78 animals without regard to individual experimental groups, time of inoculation, or other factors. An analysis of this series of animals group by group by a thoroughly satisfactory statistical method is virtually impossible, owing to the small number of animals in each group. But though the specific degree of relationship by groups cannot be obtained, it is relatively simple to show that such a relationship does or does not exist. Simple correlation coefficients were calculated for each of the 9 groups between the blood cell values before inoculation and the numerical estimate of malignancy, using both methods of estimating the tumor malignancy; the results are shown in Table III. Correlations were also made upon the 2 additional groups of 13 rabbits previously referred to; the results are also shown in Table III under the headings of Groups 10 and 11.

For our present purpose, the pre-inoculation blood cell values for each animal in the analysis given in Table III have been combined and are expressed by a single numerical value which is considered to represent "mean susceptibility." The reasons for expressing the entire 15 pre-inoculation blood cell values as a single value are as follows: first, the technical inaccuracy of the mean of any particular blood cell value may be great; second, a single such inaccuracy might

² Since the paper upon distributions (2) was published, a definite trend in certain classes of cells has occurred, necessitating slight changes in the modal values for total white cells, eosinophiles, and lymphocytes.

materially affect the correlation coefficient in groups of less than 10 counts; and finally, the mathematical processes involved in handling multiple curvilinear relationships are confusing to the non-statistical reader.

The method by which the entire 15 pre-inoculation blood cell means were combined into a single numerical value may be summarized as follows: first, the mean malignancy of the tumor for the 78 rabbits as expressed in Text-figs. 1, 2, and 3 was found to be about 58 per cent; second, an imaginary horizontal line was drawn through each chart in the text-figures at the level of 58 per cent; third, those points in the smoothed curves which fell above this imaginary line of 58 per cent were said to belong to boxes representing susceptible animals; fourth, any animal which for any given blood cell factor had a pre-inoculation mean falling into a "susceptible box," was said to be susceptible to tumor in so far as this particular blood factor was concerned; fifth, the total number of such susceptible factors was then determined for each animal. This total number was considered to be the numerical estimate of the susceptibility of the particular animal to the tumor. In Table III, the figures in the column under the heading "mean susceptibility" are the simple averages of the so called susceptible factors for each group of animals.

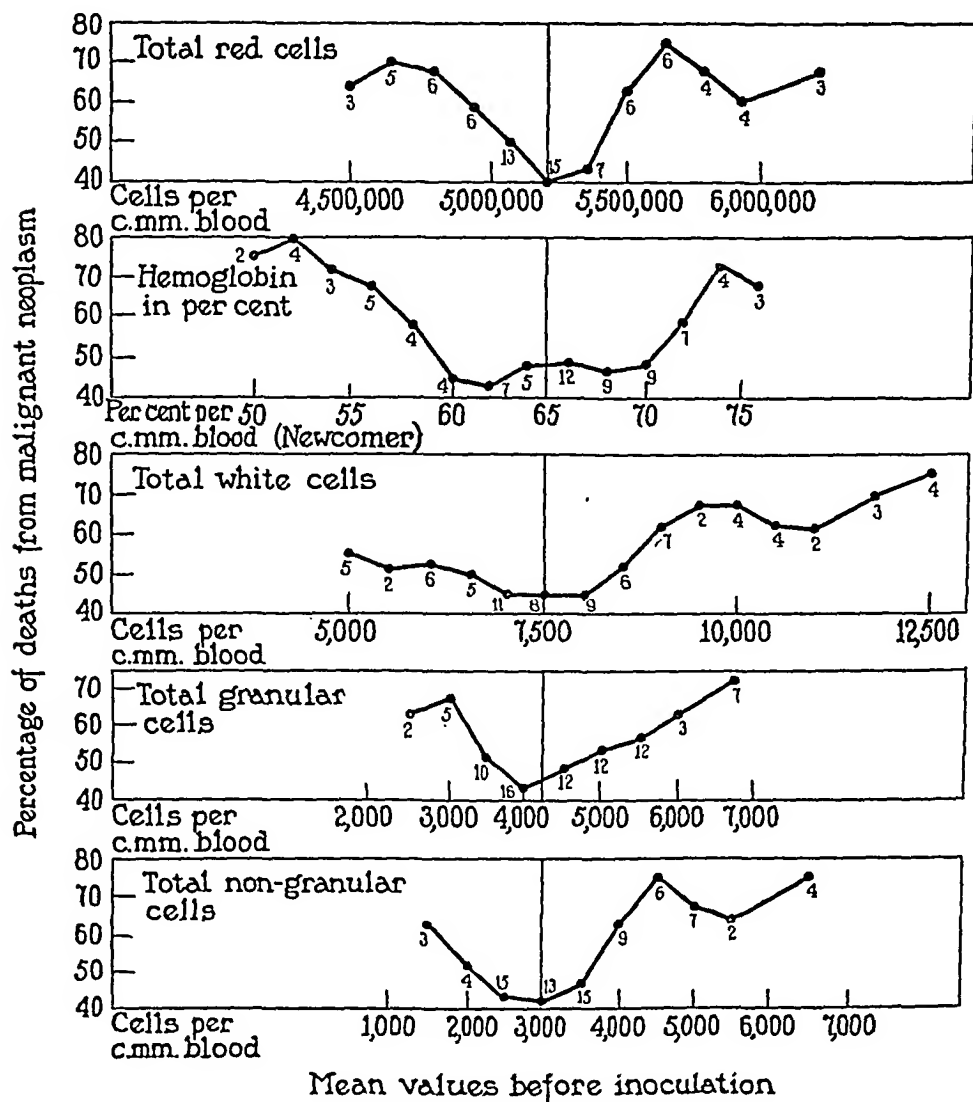
The coefficients of correlation listed at the bottom of Table III are based upon the group means given in the table and represent the relations, first, between mean susceptibility and mean malignancy, second, between mean susceptibility and mean number of foci, and third, between mean malignancy and mean number of foci respectively. A similar series of correlations upon the individual animals of each group are shown in the 3 right hand columns of Table III. Perfect correlation would be 1.000 and the absence of correlation 0.000. Neither perfect correlation nor the absence of correlation is often obtained in practice, and the coefficients obtained lie between these extreme values. The degree of correlation depends for its significance upon the number of values in the series. Since the significance of a coefficient depends upon the number of values in the series to be correlated, a correlation coefficient of $+0.300$ obtained for the entire 78 animals would be significant while one of $+0.600$ for 5 animals or less would not be significant. A coefficient of correlation is arbitrarily taken to be significant when the chances of its occurring by a random association of unrelated variables is less than 1 per 100. It is considered probably significant if the chances of its occurring by accident are from 1 to 5 per 100.

It should be noted that in the analysis contained in Table III, "deaths," "probable deaths," "probable recoveries," and "recoveries" are designated by the numbers 4, 3, 2, and 1 respectively while in the text-figures, these values are represented by 100, 67, 33, and 0 per cent. The use of numbers rather than percentage values in the analysis of Table III was solely for the sake of facility in making the calculations and in no way affects the results obtained.

RESULTS

The Relation of Blood Cell Values of Individual Animals to Malignancy

In Text-fig. 1 it will be noted that those rabbits with average pre-inoculation red blood cell counts varying between 5,000,000 to 5,500,000 per c.mm. were the most resistant animals to the tumor. The



TEXT-FIG. 1. The relation of the blood cytology before inoculation to the course of malignant disease.

mode, median, and mean of more than 1,000 counts on normal animals lie between 5,050,000 and 5,250,000 red cells per c.mm. of blood. The low point of tumor malignancy occurred among animals with 5,200,000 red cells per c.mm., and it would seem, then, that rabbits with normal red cell counts were the most resistant to this malignant disease. However, 40 per cent of the animals with normal counts before inoculation died from the tumor while not more than 70 per cent of animals with abnormal counts succumbed.

The values for the hemoglobin content show that the rabbits which were most resistant had mean pre-inoculation hemoglobin³ values of between 60 and 70 per cent (Text-fig. 1). The mean, median, and mode of the normal counts already referred to lie within these limits. Again, it would seem that rabbits with abnormal values were the most susceptible to the malignant disease. Among the animals with a normal hemoglobin content, the death rate was 45 per cent and among those with abnormal values, the actual and probable death rate was 75 per cent.

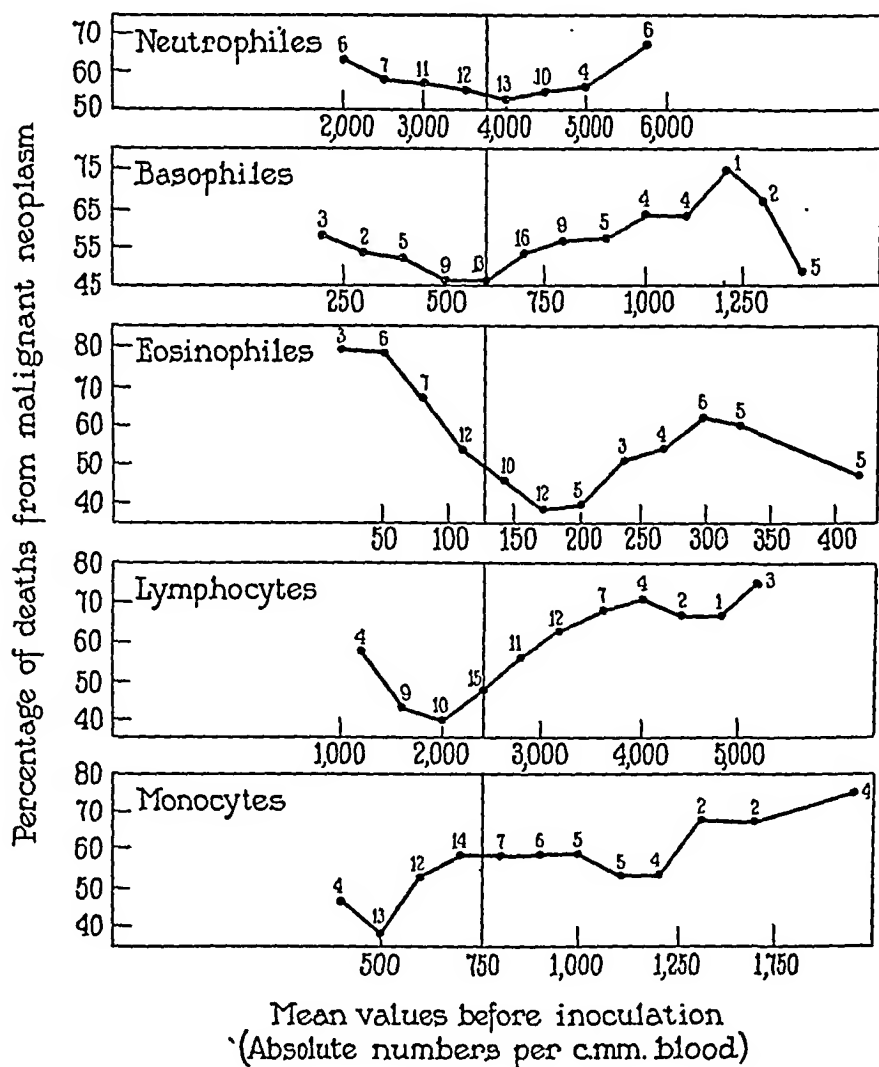
The rabbits which were most resistant had mean white blood cell counts before inoculation of between 6,500 and 8,000 per c.mm. (Text-fig. 1). The mode of the normal counts was approximately 7,500 per c.mm. Animals with mean counts below 6,500 tended to be slightly more susceptible to tumor than those within modal limits. Animals with white blood cells of more than 9,000 were definitely more susceptible than those with low and normal counts. The highest susceptibility of 70 to 75 per cent occurred in the 7 animals with total white cell counts of approximately 12,000 per c.mm.

Normal mean total granulocyte counts before inoculation were in general associated with resistance to the tumor while both high and low granulocyte counts were associated with greater susceptibility (Text-fig. 1). The rabbits which were most resistant had mean pre-inoculation granulocyte counts of between 3,750 and 4,250 cells per c.mm. These values coincide with the modal point for normal animals.

The total non-granular cells of the blood before inoculation also

³ The values for hemoglobin are only relative, due to the wide variation between methods of estimation, readings by different individuals, and with different instruments of the same type.

seemed to be related to the outcome of the malignant disease. The rabbits which were most resistant had pre-inoculation values of between 2,350 and 3,750 cells per c.mm. (Text-fig. 1). The most sus-



TEXT-FIG. 2. The relation of the blood cytology before inoculation to the course of malignant disease.

ceptible animals were those which had pre-inoculation values above 3,750 per c.mm. Increased susceptibility was also noted in animals with total non-granular cell counts of less than 2,250 per c.mm.

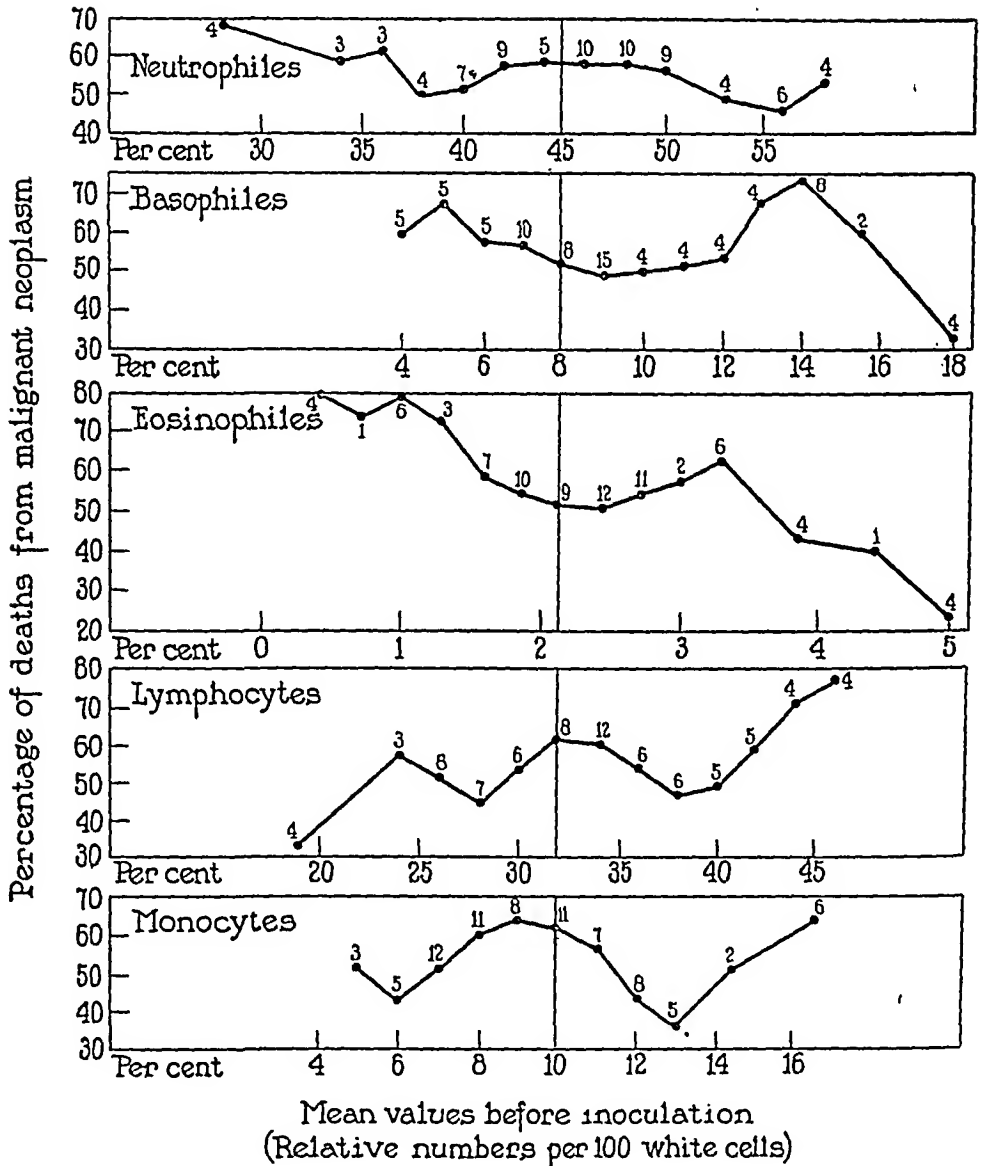
Again, deviation from the modal value seemed to be associated with lessened resistance.

Text-fig. 2 illustrates the relation of the various classes of white cells to the outcome of the malignant disease. The absolute numbers of cells per c.mm. of blood are given. It can be seen that, in general, rabbits with normal or approximately normal neutrophile, basophile, and eosinophile counts were more resistant than those with abnormal values; but it should be noted that in the case of extremely high basophile and eosinophile values, the animals tended to be resistant. In the case of the eosinophiles, however, low values were associated with an unusual susceptibility. The reaction of the various neutrophile values was of a minor order since the difference between the most susceptible and the most resistant animals was but 10 per cent.

The most resistant rabbits had pre-inoculation lymphocyte values of approximately 2,000 per c.mm.; of the 34 animals in this class, 21, or 62 per cent, were resistant to the disease. On the other hand, only 3 of the 10 animals with mean lymphocyte values of 4,000 or more per c.mm. were resistant. There was a slight tendency for animals with lymphocytes below 2,000 per c.mm. to be in the susceptible category.

In the case of the monocytes, those animals which were most resistant to the tumor had the lowest pre-inoculation values while those which were most susceptible had the highest. An exception is to be noted in the case of 4 animals with monocyte counts of below 500 per c.mm., which succumbed to the disease. The tendency for animals with low monocyte counts to be resistant and those with high counts to be susceptible is exactly the opposite to the eosinophile findings in which high values were associated with resistance and low values with susceptibility.

In Text-fig. 3, use has been made of the mean number of each white cell type per 100 cells, that is, relative values in contrast to the absolute mean numbers per c.mm. of blood previously employed. Two contrasting types of trend in relation to resistance to the tumor are to be noted. The trend of the neutrophiles, basophiles, and eosinophiles is down, that is, the more susceptible rabbits had low relative values before inoculation while the most resistant ones had high values. The trend is very similar for the three types of granular cells although it is most striking in the case of the eosinophiles. With respect to



TEXT-FIG. 3. The relation of the blood cytology before inoculation to the course of malignant disease.

the non-granular cells, however, the tendency here shown is for low values to be associated with greater resistance and high values with susceptibility. The trend in the case of the lymphocytes is in striking contrast to that of the eosinophiles. The relative monocyte values were very irregular in their relation to the outcome of the malignant disease.

It should be noted in regard to the relative values of the various white cells, as shown in Text-fig. 3, that the most resistant animals did not have counts which fell within modal limits. This is in marked contrast to the results found for the total white cell counts and those for the total granular and non-granular cells, the hemoglobin percentages, and the red cell counts, and to some extent, it is in contrast to the values for the absolute numbers of the various types of white cells (Text-figs. 1 and 2).

TABLE III

Mean Susceptibility as Determined from the Blood Cytology before Inoculation, Correlated with the Actual Course of a Malignant Neoplasm

Group	No. of animals in each group	Mean susceptibility	Mean malignancy	Mean no. of foci	'SM	'SF	'FM
1	9	3.7	3.1	7.3	+.8974	+.6825	+.8300
2	5	2.6	2.6	5.4	+.0417	-.1379	+.8539
3	9	3.7	3.0	7.0	+.6397	+.5621	+.8669
4	9	2.3	2.4	3.6	+.5086	+.5013	+.8000
5	10	1.9	2.0	3.6	+.5770	+.7484	+.9425
6	4	4.0	2.8	7.0	+.1136	-.1199	+.8992
7	12	2.8	3.1	8.0	+.8358	+.6237	+.8548
8	12	2.4	2.6	5.8	-.1267	-.0658	+.7608
9	8	2.1	2.3	4.4	+.8017	+.8610	+.8966
10	8	3.1	2.8	5.0	+.7234	+.5757	+.9397
11	5	5.0	3.4	10.0	+.8965	+.8933	+.9863
Coefficients of correlation upon the entire 91 animals..					+.6390	+.5607	+.8858*
Coefficients of correlation upon the 11 group means.					+.8577	+.8582	+.9325

r = coefficient of correlation.

S = mean susceptibility.

M = mean malignancy.

F = mean number of foci.

* Transformed method correlations (14).

The Relation of the Blood Findings to Malignancy in Groups of Animals

In Table III which contains the analysis of the material from the standpoint of animal groups, it will be seen that the correlation between mean susceptibility and mean malignancy is greater when calculated from the 11 group means (+.858) than when calculated from the individual values for the 91 animals (+.639). In addition,

the relation between mean susceptibility and mean number of foci was greater for the group means (+.858) than for the individual values (+.561). And again, the correlation of mean malignancy and mean number of foci was greater for the group (+.933) than for the individuals (+.886).

The correlation coefficients as regards pre-inoculation blood cytology (mean susceptibility) and the outcome of malignancy are comparable in magnitude with those coefficients based upon the relations between the two methods used for estimating malignancy. Both methods of estimation gave comparable results since their coefficients of correlation with the pre-inoculation blood cytology are nearly the same, namely, +.8577 and +.8582 for the 11 groups of animals and also +.6390 and +.5607 for the entire series.

DISCUSSION

No attempt has been made in this paper to give more than a brief outline of the relationships of the blood cells before inoculation to the course of the disease induced by a transplantable malignant neoplasm. The chief aim has been to show whether such relationships exist. This is the case, and it ought to be possible to predict, in the generality of cases, the eventual outcome of the disease from a cytological study of the blood before inoculation. Certain aspects of this matter and of the experimental material in relation to it will first be considered.

The Animal Material.—The use of stock rabbits of various breeds, types, and sources in these experiments was probably a benefit since a maximum number of blood variations and types of response to experimental conditions might be expected. However, the relationships encountered could conceivably be due to unknown differences in breed and type rather than to intrinsic differences in the blood formulae. In order to determine whether these relationships of pre-inoculation blood counts to resistance are dependent or independent of breed or type, other experiments with pure breeds of rabbits will have to be carried out. These are now in progress. They will serve the additional purpose of determining possible differences in cell formulae of different breeds as well as of different individuals of the same family.

The Hematological Procedure.—It is generally accepted that the numbers of blood cells in an individual animal are subject to spontaneous variations over periods of weeks or months. In attempting to relate blood findings to resistance, this feature undoubtedly plays an important rôle since an accurate blood cell formula determined upon the day of inoculation might differ significantly from one made 2, 3, or

20 weeks before inoculation. In addition, blood cell values consisting of averages of weekly determinations made during the 10 weeks preceding inoculation might differ significantly from those obtained at other intervals. The duration of the most desirable period for making the pre-inoculation counts is not known, and the appropriate number of such counts is undetermined. It seems possible, however, that with this particular tumor at least 10 counts should be made over a period of 3 weeks prior to inoculation.

The Tumor.—Any application of the results to other transplantable tumors or to the spontaneous tumors of man and the lower animals, must be made with a realization of the special material used in our experiments. From the analyses of our observations, it seems possible that a method has been evolved by means of which the cytological elements of the blood and perhaps other biometrical factors can be studied in the relation to susceptibility to transplantable tumors and other diseases. A preliminary study of such nature has already been made in experimental syphilis (16); and a more detailed report is in preparation.

In the experiments, the method of unilateral testicular inoculation was always employed. Whether the use of other sites would result in similar findings is not known. It must be borne in mind that different tumors are necessarily used as the inoculum for different experiments, and it is by no means probable that the state or condition of the tissue employed was identical in any two experiments.

The Method of Classifying Malignancy.—A satisfactory appraisal of malignancy in any given animal is admittedly difficult. The two numerical methods here employed are arbitrary and often at variance. For instance, death from inanition may result from tumor involvement of the jaw and no other tumor be found at autopsy. This might be classified as a "death" by one method or an instance of considerable resistance by another. Furthermore, an animal surviving for 2 months with widespread metastases would be described as a "probable death" while another animal dying soon after inoculation because of a single metastasis which occurred in the hypophysis would be classified as a "death." The method of counting the actual number of foci is also subject to correction since one animal with 12 foci may die in 4 weeks while another in the same group with 15 foci may live for 10 weeks. From the results of the present analyses, it seems questionable whether an animal with 10 foci is less susceptible than one with, for instance, 18 foci. It is all the more striking, therefore, that such high correlations between pre-inoculation blood findings and animal resistance have been found.

The question of the special character of the disease in relation to pre-inoculation blood cytology has also been considered, although the present material is too limited to warrant more than general suggestions. A preliminary survey, however, indicates that particular types of metastatic involvement may perhaps be associated with certain pre-inoculation blood cell values.

The Statistical Methods of Analysis.—It is quite obvious that the present problem involves multiple curvilinear correlations of a very complex sort (15). Before such an analysis can be undertaken by any of the usual methods, however, more

data are necessary in order to give approximate mathematical formulae to the curves of all the various cell values in relation to resistance. Further work in the direction of normal blood cell relationships is also indicated (7). The present paper, therefore, should not be considered as an attempt to measure the *degree* of the relationships between blood cell values and resistance, nor as an attempt to express these relationships with mathematical exactness. The present data suffice merely to show the presence and the general direction of these relationships.

The Relation of the Blood Findings to Malignancy in Individual Animals

From the results, as presented, it is impossible to determine how often an animal having one blood factor indication of susceptibility will have others giving a similar indication. The following tabulation gives an idea of the frequency with which these points or values of susceptibility occurred. The number of points of susceptibility for each animal as described in the section on material and methods, are taken from the 15 blood factors representing susceptibility as given in the curves of Text-figs. 1, 2, and 3. The entire 78 animals used in Table I plus the 2 additional groups of 13 animals mentioned in Table II are classified on the basis of the number of points of susceptibility as denoted by the pre-inoculation blood cell values. The malignancy of the tumor in each class is expressed in percentage values as previously described.

TABLE IV

Points of susceptibility	No. of animals	Proportion of resistant animals
		<i>per cent</i>
0	6	100
1	13	93
2	22	59
3	19	26
4	12	25
5	11	27
6	5	0
7	3	0
	91	

The first fact to be noted in the above tabulation is the distribution of the numbers of animals. This distribution has a decided skew to the right, indicating that the exceptional animals of the series were

more often highly susceptible than highly resistant. From this finding, it might be construed that more factors contribute toward the production of susceptibility than toward resistance.

The second point is that the blood of these animals indicated a relatively small number of points of susceptibility. Out of a possible 15 points which any animal might show, the majority had only 2 or 3 and the maximum was 7 points. A possible explanation for this finding suggests itself. The selection of normal animals for the experiments was made on the basis of weight, age, body build, and freedom from clinical signs of spontaneous disease. This implied a generally good physical condition. It might be assumed that such a healthy condition would be associated with a blood formula containing few points of susceptibility to disease, both spontaneous disease and that resulting from outside interference, such as the tumor. If this assumption is correct, it follows that rabbits with more than 7 points of susceptibility, which presumably represent a general breakdown of body resistance, would not attain the age of the animals of these experiments, or if living, would not be chosen as normal animals. This explanation is substantiated by the trends of the curves in Text-figs. 1 and 2 in which animals with normal blood values tended to be most resistant while variation either above or below normal was associated with lowered resistance to the tumor.

It is possible that susceptibility to different diseases may not be expressed by the same pre-inoculation blood formula, but by variations of a quantitative or a qualitative nature. This phase of the subject should be referred to because of the suggestion contained in the findings on the relative percentage values for the white blood cells given in Text-fig. 3. These results apparently show a certain amount of specific association in that they are independent of normal values.

The third point in the tabulation to which attention should be called is the close correlation of the number of points of susceptibility with the proportion of resistant or susceptible animals. The 6 animals which had no blood findings indicative of tumor susceptibility were entirely resistant to the disease, and the 8 animals with 6 and 7 blood factors indicative of tumor susceptibility showed little or no resistance to the tumor. In the case of the 42 animals with 3, 4, and 5 points of susceptibility, 75 per cent succumbed to the tumor. Of the 13

animals with only a single point of susceptibility, 93 per cent were resistant. The only group of animals in which it would be difficult to foretell the reaction to tumor inoculation with considerable accuracy is that with 2 points of susceptibility; 3 out of every 5 animals were resistant. This group is the largest in the series, but the succeeding one of 3 points of susceptibility is almost as large.

An index of resistance might, therefore, be devised from the above analysis in which all rabbits with 3 or more points of susceptibility are classified as susceptible and all with 2 or less, resistant. By means of such an index, 73 of the 91 animals, or 80 per cent, would be accurately classified. This accuracy can be further increased to 90 per cent for this series by the use of such quantitative biometrical methods as partial correlation. In this further analysis, account has been taken not only of the presence, but also of the degree of resistance or susceptibility as indicated by each blood factor and the relative importance of the several factors. It has been found from these calculations that certain combinations of factors seem to bear more relation to malignancy than others. These points will be discussed in subsequent papers.

The Relation of the Blood Findings to Malignancy in Groups of Animals

Probably one of the best tests of the applicability of an index of resistance derived from the values obtained on individual animals is its accuracy in representing group values. Table III represents such a test on the present material. That the coefficients of correlation upon the group values were higher than the combined coefficients upon the individual animals was to be expected; group values are less affected by errors of estimation from the standpoint of mean susceptibility, mean malignancy, and mean number of foci. The errors in calculating a coefficient based upon small numbers of animals are sometimes large as in Groups 2 and 6 with only 5 and 4 animals respectively. The very high coefficients obtained in the case of Group 11 which also had only 5 animals may be explained likewise by chance due to the small number of animals or by the fact that an especially large number of blood counts before inoculation were made.

Satisfactory coefficients of correlation depend upon accurately determined variations between individuals. In Group 8 comprising

12 rabbits, only 1 animal was a "recovery" and only 2 were "deaths." When the series came to autopsy, the distribution of living tumor in the majority of the other 9 animals was such that a classification of "probable deaths" or "probable recoveries" was unsatisfactory. It is interesting that in these particular animals, the pre-inoculation blood cell values showed few significant variations. The occurrence of such uniformity in a group with an absence of any considerable numbers of fatal or recovered cases probably explains the lack of a significant correlation. However, when these animals are considered with the rest of the 78 rabbits, either individually or as a group, their blood values are closely correlated with the course of the malignant disease. This fact is shown by the values of their group means in Table III.

SUMMARY AND CONCLUSIONS

1. The blood cytology of 91 rabbits was studied prior to inoculation with a transplantable malignant neoplasm. The following statements refer in each instance to the mean values of the pre-inoculation counts.

2. The animals which were most resistant to the malignant disease had, before inoculation, normal red and white cell counts, normal hemoglobin percentages, high eosinophile counts, and low counts of monocytes and lymphocytes. The relations of the neutrophile and basophile counts were irregular, but normal values also appeared to be associated with greater resistance.

3. The most susceptible animals were those which had, before inoculation, red cell counts above 5,500,000 or below 5,000,000 per c.mm.; hemoglobin above 70 per cent or below 60 per cent (New-comer); white cell counts below 6,000 or above 8,500 per c.mm.; low eosinophile, high monocyte, or high lymphocyte counts.

4. No animal with any of the following findings prior to inoculation recovered completely from the tumor as determined by autopsy examination: red cells above 5,500,000 per c.mm. of blood; hemoglobin above 70 per cent; total white cells above 10,000 per c.mm.; eosinophiles below 120 per c.mm., or below the relative value of 1.5 per cent; basophiles below 400 per c.mm., or below the relative value of 6 per cent; lymphocytes above 3,600 per c.mm.; monocytes above 1,500 per c.mm.; neutrophiles above 5,000 per c.mm.; and total granular cells above 5,700 per c.mm.

In the case of each of the following pre-inoculation values, only 1 animal was completely free from tumor at autopsy: hemoglobin below 60 per cent; red cells below 4,800,000 per c.mm.; total granular cells below 3,300 per c.mm.; total non-granular cells below 2,300 per c.mm.; total non-granular cells above 3,700 per c.mm. No animal with pre-inoculation eosinophiles above 3.9 per cent, or basophiles above 16 per cent died from the tumor.

5. The blood findings before inoculation could be related to the character and outcome of the malignant disease, from the standpoint of animal groups as well as in the case of individual rabbits.

6. From the results of the experiments here reported, it seems possible to predict with an accuracy of between 80 and 90 per cent the individual resistance or susceptibility of rabbits to the tumor by a study of their blood cells before inoculation.

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THE EFFECT OF TESTICLE EXTRACT AND OF NORMAL SERUM ON A TRANSPLANTABLE EPITHELIAL TUMOR OF THE RABBIT¹

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PLATES 48 AND 49

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In the course of some experiments designed for comparison of the properties of the filtrable agents of chicken tumors with those of typical viruses, it was noted that when the tumor agent was brought in contact *in vitro* with those tissues of susceptible animals in which the tumor grows well, for example, muscle, it was adsorbed and fixed—or inactivated—while similar tissues from non-susceptible fowls or other animal species were inert in this respect (1). On the other hand, vaccine virus, which thrives in the testicle, was not inactivated by contact *in vitro* with testicle extract, but had its infectivity enormously increased by this treatment (2). This phenomenon, the enhancement of infectivity by testicle extract, has thus far proved to be universal for a great variety of viruses and bacteria (3). Work has recently been done tending to prove that the testicle extract acts by causing an increase in tissue permeability (4). The present study deals with the effect of testicle extract on the growth of a transplantable carcinoma of the rabbit, its action being contrasted with that of normal serum, which ordinarily interferes with the activity of most infective agents.

Material and Method

The Brown-Pearce epithelioma of the rabbit, a tumor having its origin in the hair sheaths of the scrotum (5), has been used for these experiments. This tumor

¹ A preliminary report of this work appeared in *Science*, 1930, 72, 603.

² This investigation was carried out by means of funds from the Rutherford Donation.

has the peculiarity that progressive growth rarely results from inoculations in locations other than the testicle and brain. Subcutaneous or intracutaneous grafts will grow for a time but almost invariably retrogress, a fact which is difficult to explain, considering that a testicle graft often gives widespread metastases to the skin.

The rabbit tumor is prepared by chopping finely and then squeezing through gauze in order to get an even suspension of the cells. Before inoculation the material is diluted to two to three times its volume with Ringer's solution. The testicle extract is prepared by grinding the pure glandular tissue of rat, rabbit or bull testicle with sand and an equal volume of Ringer's solution. The mixture is centrifuged to remove the cell debris and sand. Extreme precautions against contamination must be observed throughout, to exclude incidental infection which would be enhanced by the extract.

Susceptibility of Rabbits to Intracutaneous Inoculations of the Tumor.—Preliminary tests showed that if a rich cell suspension of healthy, primary tumor or its metastases was used for the intradermal inoculations, as many as twelve such inoculations could be made in a single rabbit yielding tumors of approximately uniform size. Most of the skin tumors retrogress after 2 weeks of steady growth, during which they reach a diameter of about 3 cm.

TABLE I

Tumor cell suspension plus	No. of inoculations	Larger growth than control	Same growth as control	Smaller growth than control	No growth
Rat testicle extract.....	16	0	0	2	14
Rabbit testicle extract.....	16	0	1	6	9
Rabbit serum.....	32	19	10	3	0
Ringer's solution (control).....	20				0

Action of Rat and Rabbit Testicle Extract and Rabbit Serum on the Rabbit Tumor

Thick cell suspensions were prepared from the rabbit tumor as described above. These were divided into four portions. To each portion was added respectively one of the following solutions: rat testicle extract, rabbit testicle extract, serum from normal rabbits or Ringer's solution. These suspensions were kept in tubes at 37°C. for from 2 to 3½ hours, during which period the tubes were shaken several times. After the incubation 1 cc. of each mixture was injected intradermally, two or more inoculations of each mixture being given to each rabbit. By this system each rabbit carried all of the tests as well as the control, thus ruling out the disturbing factor of individual differences in susceptibility. The results of five experiments, in which ten rabbits were inoculated in from eight to ten different areas each, are summarized in Table I.

As a parallel to the above experiment, eight animals were inoculated in the testicle with a mixture of tumor cell suspension and rat testicle extract. This was controlled by another group of eight rabbits inoculated in the testicle with the same cell suspensions diluted with Ringer's solution, and kept under the same conditions. The results showed some indication of inhibition of growth by the testicle extract.

The above experiments show conclusively that both rat and rabbit testicle extracts interfere with or even inhibit altogether the growth of the rabbit epithelioma in the skin. On the other hand, normal rabbit serum seems just as definitely to augment the growth of the tumor cells (Figs. 1 to 4). When the tumor and testicle extract mixture is inoculated into the testicle there is some indication of inhibition, but it is not so definite as on intradermal inoculation.

The Effect on Tumor Cells of Fresh Bull Testicle Extract and a Purified Fraction.—There is no indication in the foregoing experiments that the factor in the testicle extract inhibiting the growth of the rabbit tumor is identical with that which enhances infections and increases the permeability of tissues. In collaboration with Claude and Helmer, the fraction of testicle extract carrying the enhancing factor has been carried to a high degree of purity.³ The entire enhancing value of 100 gm. of bull testicle has been isolated in 0.4 gm. of the purified fraction, a stable material with a very low protein content. This fraction retains the spreading power for inert particles. In the next group of experiments a comparison has been made of the action of fresh bull testicle extract with that of the purified infection-enhancing factor.

Experiment.—Five experiments were made using the same methods as described above, the test materials being the full extract and the purified fraction of bull testicle. In four of the experiments the rabbit tumor and the solution were kept in contact for from 1 to 2 hours at room temperature before inoculation into rabbits, and in the fifth experiment they were inoculated immediately after mixing. The results are summarized in Table II.

Comparing the results of these experiments with those shown in Table I it will be noted that while fresh bull testicle extract has a definite inhibiting action on the tumor, it is not quite as active as the extract of rabbit or rat testicle. The length of contact before inoculation seems to be of no importance. The purified fraction gives even more pronounced results than the full extract. While this

³ The details of this technique will be given in another paper.

finding does not establish the point indubitably, it suggests strongly that the factor in the testicle extract which is responsible for the enhancement of infections is also active in inhibiting the growth of the rabbit tumor.

Effect of Dilution of the Testicle Extract.—In order to determine the effective strength of the testicle extract the consequences of dilution were studied.

Rabbit tumor cells were kept in contact for 2½ hours with undiluted rabbit testicle extract and with the extract diluted 1:10, 1:100 and 1:1000. At dilutions of 1:100 or over the inhibiting action was no longer evident. The same result was obtained in two other experiments in which bull testicle extract was used. The findings in a typical experiment are shown in Table III.

TABLE II

Tumor cell suspension plus	No. of inoculations	Larger growth than control	Same growth as control	Smaller growth than control	No growth
Fresh bull testicle extract.....	8	0	0	8	0
Purified bull testicle extract.....	36	0	2	20	14
Ringer's solution (control).....	44				0

TABLE III

Tumor cells plus rabbit testicle extract	No. of tests	Larger growth than control	Same growth as control	Smaller growth than control	No growth
Undiluted	2	0	0	0	2
1:10	2	0	0	1	1
1:100	2	0	2	0	0
1:1000	3	0	3	0	0

In two instances in which the rabbits were inoculated in the skin with a mixture of tumor cells and diluted testicle extract, the tumor did not retrogress as it almost always does when inoculated alone, but continued to grow and gave widespread metastases. This suggests the possibility that the diluted extract may act as a stimulant.

DISCUSSION

The experiments here reported show that a factor existing in rat, rabbit and bull testicle extracts strongly interferes with or altogether

inhibits the growth of a transplantable rabbit epithelioma. These findings are in strong contrast with those on the influence of the same organ components upon bacterial or virus infections. Whether the factor adversely affecting tumor is the same that enhances infections and increases the area of spread of injected material has not yet been absolutely determined. However, the fact that the highly purified enhancing factor from bull testicle manifests both spreading power and an inhibiting influence on rabbit tumor makes it seem probable that the same factor is responsible for all of these manifestations. The evidence so far gathered suggests that the increased tissue and probably cell permeability (6) brought about by the active testicle extract is the mechanism behind the phenomenon. The relation of this factor to certain other activities of testicle extract, the stimulation of normal and malignant cell multiplication *in vitro* (7) and *in vivo* (8), is as yet unestablished.

SUMMARY

Extract of rat, rabbit or bull testicle prevents or retards the growth of a rabbit tumor when a mixture of the extract and a tumor cell suspension is inoculated intradermally. Similar mixtures, made with normal rabbit serum instead of testicle extract, give rise to tumors which grow with unusual rapidity. The results are the opposite of those obtained with pathogenic bacteria or filtrable viruses which are enhanced by testicle extract and generally inhibited by normal serum.

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EXPLANATION OF PLATES

PLATE 48

FIG. 1. *Right side.* (1) and (2) Tumors resulting from intradermal inoculation of tumor cell suspension diluted with Ringer's solution. (3) and (4) Tumors resulting from inoculation of tumor cells and testicle extract.

FIG. 2. *Left side.* Results of inoculation in four areas of tumor cells mixed with rabbit serum.

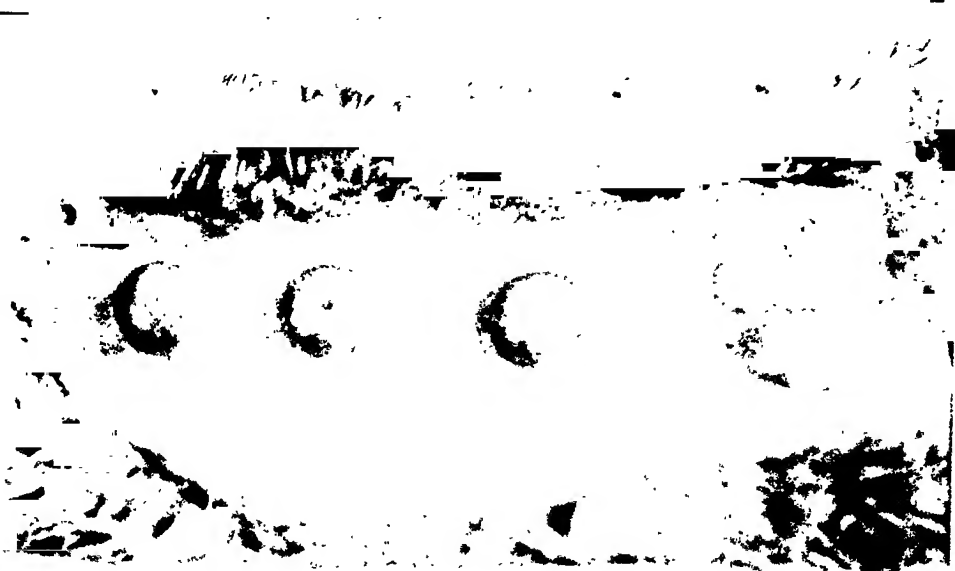
PLATE 49

FIG. 3. *Right side.* (1) and (2) Results of intradermal inoculation of tumor cell suspension + rat testicle extract. (3) and (4) Results of inoculation of tumor cell suspension + rabbit testicle extract. (5) and (6) Results of inoculation of tumor cell suspension + Ringer's solution.

FIG. 4. *Left side.* Results of inoculation of tumor cell suspension + rabbit serum in the four areas.



1



2

Young mice of 17 to 19 gm. were used. They had free access to water at all times and remained lively throughout the thinning period of 7 to 9 days. When the weight had fallen to 10 to 12 gm., dye was injected. In type experiments Chicago blue 6B was used in a half strength solution isotonic with the blood (a watery 17.0 per cent¹ solution mixed with an equal quantity of Locke's solution), 0.08 to 0.1 cc. being given in the course of a minute. Decapitation was done 6 to 10 minutes later, the animal was pinned on a board at once, and a skin flap everted. The hair was oiled where it was to be cut through, in order to prevent a scattering of ends on the exposed surface. With blunt scissors a first slit was made along one side in the axillary line and this was extended transversely across the upper and the lower abdomen to the other side. The flap was freed from the web-like underlying tissue with the scissors, and everted and brought in contact with a piece of microscope slide, to which its moist surface at once adhered. Special care was taken to prevent distortion or stretching. The preparation could now be inspected under the binocular dissecting microscope by the cooled light used in our previous work (1). The hair provided an underlying ground of white against which the local color differences stood forth brilliantly. Even the gentlest shaving before injection of the dye resulted in much disturbance of the local circulation, with not a few vascular areas closed off, and irregular staining.

Chicago blue 6B is a poorly diffusible dye (1), as evidenced *in vivo* by its tardy escape from the vessels and its slow secondary spread. It colors the mouse much faster than the rabbit, yet a considerable period elapses before the skin becomes evenly stained. In mice killed 6 to 10 minutes after injection the everted flap has a remarkable appearance (Fig. 1). It is everywhere stippled with small blue dots on a ground which is unstained, appearing white because of the hair beneath. The dots are 0.1 to 0.2 mm. across, 1/2 to 1.0 mm. apart, and in the midst of each ramifies a solitary, contorted, venular tree (Figs. 1, 3, and 4). Some of the corresponding arterioles still contain sufficient dye for the fact to be made out that they are interspersed between the venules with notable regularity, and that each passes to the center of an unstained area (Figs. 3 and 4). The colored dots are most widely separated in the neighborhood of the midline, where vessels in general are fewest and the staining least. Here also the muscle of the panniculus carnosus is practically non-existent (Fig. 6), whereas toward the sides it thickens, as does the connective tissue also, and some general staining is superimposed upon the dotting. In the thinned mice fat has practically disappeared from the skin, and, looking at its under side, one can make out the butts of the hairs, in short, irregular ranks of special whiteness (Fig. 3).

In animals allowed to live more than 10 minutes after the dye injection, some general staining of the corium takes place. This does not come about by an enlargement and coalescence of the colored dots. They remain discrete but are

¹ Much more of the purified material now employed is required to make an isotonic solution than of that used in our previous studies.

gradually lost in an even coloration, the result of an escape of dye from the capillaries everywhere (Fig. 2).

Conditions Localizing the Escape

The spread of Chicago blue through the cutaneous tissue after death is so slow that differences in its local distribution can be scrutinized at leisure. Under the microscope one sees on the under surface of the skin, slightly to one side of the midline, a tracery of fine, roughly parallel blue lines,—the dye-containing capillaries supplying the scanty fibres of the panniculus carnosus (Fig. 5). Focussing shows that the perivenular spots of color lie beyond, in the corium. The arterioles supplying the latter pass almost vertically to it, and so also with the corresponding venules. Between the vessels of the two sorts only capillary channels can be made out in the corium, and these but occasionally since most are empty of the dye-stained blood. Cleared preparations from mice injected with India ink gelatin mass (Fig. 5) show the vascular arrangement excellently described by Kreyberg for the skin of the back (3). Separate arterial and venous networks beneath the panniculus connect with the capillaries of this muscle sheet and send off branches that pierce it to join other, finer-meshed, networks just superficial to it. From these in turn small, solitary arterioles and venules pass at almost regular intervals to the capillary network in the corium. The staining with Chicago blue is localized to the tissue in the neighborhood of these final venules. Hence the dotting with color seen in the gross.

The singular localization of the staining suggests the probability that there are special structures for which the dye has an affinity. But microscopic sections stained with eosin and methylene blue (or hematoxylin) have failed to disclose any such. The mammary gland lies beneath the panniculus (4) and can be ruled from account. The hair follicles fail to "take" the vital dyes, their arrangement has no relation to the dotting with color, and except for them the stratum composing the corium appears to have the same general texture everywhere in the region studied (Fig. 6). The subcutaneous fatty layer had practically disappeared in our thin animals; and it does not stain, nor is it so situated as to be responsible for the localization of color. Even in well nourished mice there is almost no fat in the corium itself

of the abdominal region. Furthermore the edges of the colored dots are ill defined, fading off gradually; and all dye can be forced out of the corium by gentle pressure, leaving it unstained,—facts both which indicate that it is dissolved in the tissue fluids. And finally, dotting of the same sort is produced with other dyes fitted by their rate of diffusibility to disclose it, namely, pontamine blue, trypan red, and brom phenol blue. True, the capillaries are so permeable to this last that it must be injected very rapidly and the corium examined practically at once if the dotting is to be perceived amidst an intensifying general coloration; while with trypan red the examination cannot be much deferred. But these differences are obviously dependent, like those in voluntary muscle (1), upon the degrees of diffusibility of the dye stuffs.

Can local vascular differences be responsible for the stippling of the corium with color? One such difference is very evident. The contorted, venular trees offer a far greater surface for escape of dye from the blood than exists elsewhere. But the dye is not narrowly localized next the venular wall nor does it extend therefrom secondarily to form the spots of color. These arise *in situ* and they extend considerably beyond the region in which the venular tree is situated. To explain their relatively large dimensions one must suppose some dye to have got out from the portion of the capillaries adjoining the venules. Injected specimens show that in the neighborhood of the latter the capillary web is not especially abundant, nor are the channels wider there (Fig. 5).

The Escape into the Skin of the Ear

It is impossible to watch directly the spread of dyes from the blood into the skin of the body of the living mouse. But the ear presents excellent opportunities for this.

The mouse ear offers great advantages for the study of vascular phenomena, yet it has been little utilized. Leonard Hill has made pressure experiments upon it (5); and Lehmann (6) states that he had been able to see trypanosomes in the blood passing through it. Fröhlich and Zak (7) give the only description of the arrangement of the vessels that we have been able to find; but, while adequate for their purposes, it does not provide the information necessary for ours.

Young mice of 14 to 20 gm. have more transparent ears than older ones, while in the latter, furthermore, ecchymoses of stain often develop, traceable to injuries

not easily recognized beforehand. A young animal of 20 gm. injected into a tail vein with 0.08 to 0.1 cc. of half strength Chicago blue solution during the course of a minute, manifests no symptoms, but the ear becomes pale azure, with thread-like dark vessels sharply outlined under the microscope (Fig. 9). They stand forth against a background as of shimmering ground glass (the underlying fat). After 2 to 3 minutes a patchy coloration begins of the tissue about some of the vessels, and within 5 minutes this has become marked (Fig. 10). With the binocular microscope one can see that the dye has passed out into the tissue traversed by the further portion of the capillary web and the smallest collecting venules, while no staining whatever has occurred as yet in the region occupied by the proximal part of the web and the arteriolar ramifications.

Microscopic observations are best made under ether or luminal, with the mouse so arranged on its belly in a shallow trough of plasticine that the ear stands free and vessels leading to it are not pressed upon. The hair need not be removed, but the skin should be oiled with neutral paraffin oil. Under the ear at a distance of 2 or 3 mm. is fixed an ovoid or triangular plate of opaque, white glass on a dais of plasticine, and the light of the arc is directed obliquely to illumine both glass and ear. In the combination of transmitted and reflected light thus provided the spread of dyes from the blood can be readily followed. For photographic purposes the ear is momentarily flattened in oil between the white plate and a coverslip (Figs. 9 and 10). Under such circumstances, the passage of individual red cells along the capillaries is easily discerned. But even the slight pressure of the coverslip alters the rate of staining and should be avoided.

After an injection of Chicago blue the vessels do not dilate, but the stained blood gives them almost the sharpness of a woodcut. The larger ones, veins and arteries together, radiate in a fan on the upper side of the ear, breaking up near its margin into a multitude of fine branches so interlaced that here it is often difficult to determine the type of vessel from which the dye first escapes. Between the sticks of the fan, though, the situation is better defined. One sees solitary arterioles and venules (Figs. 9 and 10) with only capillaries in the tissue between, indistinct faint blue lines which bend and loop. The posterior quadrant of the ear yields the clearest pictures.

The first escape of dye, occurring 2 to 3 minutes after the injection, stains the tissue lying immediately about the least venules; but almost as it does so the adjacent tissue turns blue too, so that an irregular colored strip is formed, with fading margins and the venule at its center. There can be no doubt, however, that extravascular dye is first perceptible in the tissue immediately next the venules; but it undergoes no evident lateral spread. After 15 minutes the strips are much more intensely blue than at first and definitely larger. In the gross the ear appears brilliantly patched with blue. Each patch or

strip can be seen with the microscope to be separated from the nearest dye-containing arteriole by a region of unstained tissue, supplied by capillaries only, which is often nearly 1 mm. across but averages about 1/2 mm. (Fig. 10). Such regions become diffusely stained later, but the strips of intense blue are often discernible for more than an hour amidst the deepening color.

Structure of the Ear

The precise situation of the dye can be readily determined. Stained cross-sections show that the ear has a central lamella of cartilage with a fatty layer to either side, overlain by corium and epidermis (Figs. 7 and 8). Amidst the fat on the outer surface are irregular aggregates of short voluntary muscle fibres. These form an almost continuous flaring layer near the base of the ear but further out are scattered, and in the region we have studied, near the margin, are wholly lacking. The fan of large vessels lies on the outer side just under the corium. If a patchily stained ear is cut off, seized at its base with forceps and pulled apart, it separates into two layers, the outer, thinner one consisting only of corium and epidermis with some scattered, easily recognizable fat cells and the fan of vessels. The relation of the patchy staining to these last remains unchanged, and one perceives that the coloration is limited to the corium. Or the intact ear can be cut across with a sharp razor and inspected microscopically, edge on, in paraffin oil. One sees then that the fatty tissue and epidermis have not stained, and the cartilage only where it is thickest, and there lightly and evenly. The blue patches lie entirely in the corium. Hair follicles are equally numerous in the patched and unstained regions. They have not colored. Staining is least pronounced near the margin of the ear, because here the corium is thin and the follicles numerous.

The fatty layer on the inner (under) side of the cartilaginous lamella is shallow and incomplete, and the corium appears thick in comparison with it (Figs. 7 and 8). Both are supplied by derivatives from the fan vessels which pierce the cartilage vertically here and there to ramify and form wide meshed networks beneath it. When the ear of the living animal is viewed on its inner side, arteries and veins are seen to emerge together through a brilliantly translucent partition

as of minute glass beads (the cartilage), and to branch like spider angiomata or contorted dendrites. Here again a blue patching can be seen to develop after the injection of Chicago blue, and one observes that it is localized to the tissue in the neighborhood of the venules.

The ears of mice injected with a gelatin mass containing India ink show far more vessels than Chicago blue renders visible (Figs. 11 and 12). Excellent preparations can be obtained when the aorta and vena cava are clamped off above the diaphragm just before the vessels of the head are washed out and injected by way of a cannula passed up into the aorta through the left ventricle. Not only is each large vein in the fan-work accompanied by an artery, but so too with most of the secondary and even tertiary branchings. Between the sticks of the fan, however, are some regions supplied and drained by solitary vessels; but such regions are far fewer and smaller than one would expect from the study of living animals with dye-stained blood. When the ears of these are closely scrutinized, one finds many regions in which there is no blood flow, the red cells being stationary where present at all. The smaller venules from such regions are devoid of dye. Evidently circulation is not taking place through the entire vascular bed but is much restricted, as is normally the case in the ears of the dog, cat, and rabbit (8). If an India ink suspension is rapidly thrown into the blood and its advancing dark columns are followed, one sees that they go, not from the arterioles to the nearest venules, but across gaps about 0.5 mm. wide bridged only by capillaries.

No arteriovenous anastomoses such as exist in some other species (9) have been detected anywhere in mouse ears cleared after injection with gelatin mass containing India ink; nor does dye or ink injection during life disclose the presence of any, even when the distribution of these substances has been slowed by pressure on the vessels at the base of the ear. Such anastomoses, if they exist, play no part in the phenomena with which this paper is concerned.

As already stated, the main vessels of the ear are situated on its outer side. In proportion as they branch they tend to become more superficial, and the smaller ones, which supply the corium with capillaries, lie next this layer or within it (Figs. 7 and 8). Capillaries are given off also to the fat, but with these we are not concerned since the adipose tissue shows no color; and there are deep ones supplying the voluntary muscle. The muscle capillaries are easily recognizable as grids of crowded, minute, parallel vessels (Fig. 11). They are definitely less permeable to dyes than the capillaries of the corium, but are so close set and numerous that the stained blood within them causes darkenings near the middle of the ear (Figs. 13 and 14). The anterior third of the organ is thicker than the rest, more hairy, and possesses in the corium a meshwork of large, very permeable capillaries. This region we have not studied.

In vitally stained mice the arterioles can be traced to their tips because they lie in tissue that for long is unstained. Their lumen is reduced to even less than capil-

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In vitally stained mice the arterioles can be traced to their tips because they lie in tissue that for long is unstained. Their lumen is reduced to even less than capil-

lary size before they break up. The open capillaries are from 3μ to 10μ in diameter, averaging 6μ , the first venules 6 to 18μ , the next larger 37μ as an average, the branches into which they give 60 to 75μ , the primary branches 75 to 150μ , and the large, fan veins 100 to 175μ . Even the largest veins are notably thin-walled, and those from which staining occurs show only a single layer of endothelial cells, in ordinary cross-sections.

The capillaries of the corium are no more numerous near the venules than elsewhere, and they first undergo enlargement when entering these vessels, as can be well seen where they enter at right angles. Where the venule continues the direction of the capillary, it is impossible to say precisely where one begins and the other leaves off.

As this description brings out, the vascularization of the corium of the ear differs from that of the body only to the extent necessitated by the shape of the organ. The vessels of the ear lie much more nearly in one plane because of the general flattening. The arterioles and venules do not rise almost vertically toward the surface and branch to all sides but ramify in the direction of the margin of the ear. Hence, as vital staining takes place with Chicago blue, one sees strips of color form, not rounded dots such as appear in the skin of the body. But the difference is merely superficial. The localized staining is traceable in both cases to a special escape of dye from the blood in the region of the venules and the further portion of the capillaries. The same holds true with other dyes, pontamine blue, trypan red, trypan blue, brom phenol blue (Fig. 13), and even the very highly diffusible patent blue V (Fig. 14). All were injected in isotonic solution (1). The variety of the dyes, and the ease with which all color can be forced from the stained tissue by general pressure rule out a fixation upon the structures of the ear.

The Gradient of Vascular Permeability

The venular trees are broader and more contorted in the ear than in the skin of the abdomen, sometimes actually corkscrewed, and their numerous, fine branches are like widened capillaries. They provide a relatively great expanse of wall through which escape can take place from the blood. That the local conditions are especially favorable to this is shown by the course of the staining, as already described. Pontamine blue escapes from only the smallest of the venules, but Chicago blue, which is more diffusible, passes through the

walls of the veins of next larger size as well, and trypan red, brom phenol blue, and patent blue V get out of veins of still greater magnitude.

Can the patching with dye be explained entirely by an escape from the venules, with secondary distribution through the tissue? The differing course of events with dyes of graded diffusibility provides the answer to this question.

Pontamine sky blue, the most indiffusible of the dyes mentioned, passes out of the blood more slowly than Chicago blue, the patching is more closely localized next the venular trees, and the generalized staining in which the patches eventually are lost takes longer to develop. The dye should be injected in an isotonic half strength solution (a 10.8 per cent watery solution mixed with an equal quantity of Locke's solution (10)), 0.1 cc. for a 20 gm. mouse. The relatively diffusible brom phenol blue (1) must be put into circulation quickly if sufficient is to reach the venules for local differences in the staining of the corium to be perceptible. When given gradually so much passes out from the capillaries that no intenser staining can take place further on from the depleted blood. When quickly injected (0.15 cc. of a 4 per cent watery solution for a 20 gm. mouse) some pale, general staining of the ear appears within a minute, and superimposed upon this a deeper patching round about the venular trees (Fig. 13). The patches are broader than with Chicago blue or pontamine blue, and, as in the case of these dyes, they develop *in situ*, not by lateral spread from the venules. In comparison with them the tissue occupied by the proximal capillary meshes and arterioles appears almost unstained. After less than five minutes the coloration has become uniform throughout the ear. When the very highly diffusible patent blue V (0.15 cc. of an 8 per cent solution for 20 gm. of mouse) has been thrown abruptly into the blood the ear rapidly becomes blue everywhere; but patches of intenser color develop with venular trees in their midst, despite the fact that the plasma has lost much of its dye along the capillary way (Fig. 14). After about 2 minutes the patching is lost in a general staining.

The changes are slower in etherized animals. Tests have shown that the differences with the dyes are not referable to differences in dosage.

The patching caused by the highly diffusible dyes cannot be the result of lateral spread from the venules, since it develops *in situ* and very rapidly. It is soon lost in a general staining. The capillaries are evidently permeable to such dyes throughout their length, but most permeable in their further portion.

Several facts speak for a permeability of the venule wall exceeding that anywhere along the capillary, and for an increasing permeability of the wall of the latter as the vein is neared. When pontamine blue has been introduced into the blood the tissue supplied from the proximal part of the capillary network remains unstained for about 15 minutes after a brilliant blue patching has appeared in the venular region. With Chicago blue the period is less; while with the other dyes a general staining develops within 2 or 3 minutes at most, the time varying with their diffusibility. In the case of poorly diffusible dyes (pontamine blue, Chicago blue) the local differences mentioned persist far too long to be explicable on the basis of relative amounts of vessel wall through which dye can escape; and with the highly diffusible patent blue V this factor would seem to be more than counterbalanced by a great loss of the pigment from the blood as it flows along the capillary way, a loss plainly evident in the relatively light hue of the contents of the venules,—from which vessels, nevertheless, escape is most abundant (Fig. 14). The fact has already been mentioned that the capillaries, while exhibiting great individual variation in calibre, do not increase in width on the way to the venule. Nevertheless with a dye of medium diffusibility (brom phenol blue) a graded increase in tissue staining takes place along the greater part of this way. With less diffusible dyes a similar gradation is limited to the distal portion of the meshwork; while with the very highly diffusible patent blue V the rapidly diminishing concentration of the dye as the blood flows along the capillary sufficiently accounts for the lack of a graded staining save near the venules where the opportunity for passage into the tissues is especially good. The even staining with all the dyes, that succeeds upon the patching, is sufficient evidence that there is plenty of stainable corium everywhere.

In the course of work to be reported later with Dr. McMaster and Dr. Hudack the circulation to the mouse ear was temporarily cut off without damage by compressing the large vessels at its base between

a glass plate and a narrow, sausage-shaped, collodion bag. After this had been done, Chicago blue was injected, a minute or two allowed for it to distribute itself in the blood, and then the circulation was permitted to reenter the ear. Vascular relaxation had occurred as the result of anoxemia and the entire network filled at once with dark blue blood. Immediately that this happened all flow was cut off again. And now in the course of a few minutes a light staining took place with the usual patchy distribution. One could see that the plasma in the venules lost color first,—despite the relatively unfavorable ratio of wall area to vessel content,—with decolorization in the adjoining capillary region a little after. The blood in the proximal part of the capillary meshwork and in the arterioles,—which did not contract,—was still dark blue.

It seems certain from these highly various observations that the opportunity for escape from the blood increases considerably along the capillaries but is greatest in the venular region.

Most of the substances carried by the blood are more diffusible than patent blue V, the best in this respect of the dyes used (1); but the serum proteins are considerably less diffusible than pontamine blue, which stands at the other end of our series. Yet serum proteins normally pass from the blood in no inconsiderable quantity, and are found to the amount of 1 to 2 per cent in the lymph from an extremity (11). Dr. Heidelberger has kindly provided us with two colored azo-albumins, a red compound (12) and a green one, for utilization in tests of the permeability of the cutaneous vessels. The green azo compound caused vascular injury, as shown by ecchymotic staining; but the red one produced no symptoms nor evident lesions, even when concentrated to a 9 per cent solution and injected to the amount of 0.2 cc. The ear of the injected animal became gradually and evenly pink in the course of the succeeding half-hour. Since all free dye had not been removed from the preparation, and there had been abundant time for a secondary distribution of the coloring matter, the finding was uncertain in its implications. For this reason Chicago blue 6B was linked with egg albumen in our laboratory, the last trace of free dye removed, and concentration effected in isotonic saline by differential filtration under pressure through a collodion membrane permeable to the dye but not to its combination with protein. The mate-

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rial thus procured was far inferior in tinctorial value to Chicago blue and yielded only an equivocal extravascular coloration.

DISCUSSION

In voluntary muscle and in frog skin the arrangement of the vessels is so orderly that local differences in the opportunity for dyes to escape along them find almost diagrammatic expression in colored patterns. In mammalian skin the vascularization is far less regular yet the findings prove that the opportunity for dyes to pass into the tissues increases along the further part of the capillaries and is greatest in the region of the venules. Here poorly diffusible substances mostly escape and the passage outwards of rapidly diffusible ones takes place most readily. That intrinsic local differences in the permeability of the vascular endothelium are responsible for the gradient of vascular permeability cannot immediately be concluded, however; for the fabric just outside may conceivably act as a reinforcing wall, local variations in it conditioning exchange. This possibility has been excluded for the ear vessels by work to be reported later.

That exchange between blood and tissues may take place in part through the walls of venules has long been inferred on anatomical grounds. As far back as 1896 (13) Starling wrote that "the production of tissue fluid is limited to the region of the capillaries and small venules." Krogh has pointed out that in human skin there are practically no capillaries except those in the papillae (8), and he infers that exchange with the tissues must take place mainly through the walls of the venules which he aptly terms "giant capillaries." Lewis (14) believes that all of the small vessels are implicated in this activity and Kreyberg (3) has stated a similar view as concerns the vessels of the skin of the mouse. The significance of our venular findings lies in the evidence they afford that the opportunity for substances to escape from the blood into the tissues is not merely as good in the venules as in the capillaries but far better. In man the cutaneous venules are developed at the expense of the capillaries (15), while even in the mouse their shape, broad and contorted, is manifestly unnecessary to the mere collection of blood. The inference seem justified that the venules are differentiated for special purposes; and the known functions of the skin provide a clue to these. They demand not only

an abundant vascularization, with a highly complex controlling mechanism, as Lewis has pointed out (14), but conditions which can be provided only by broad, thin-walled vessels with a slow current.

The skin is a protective organ continually subject to slight traumata and to local infections. Cohnheim (16) noted that leukocytes get out much sooner and easier from venules than from capillaries; and Tannenbergh (17), extending the observation, has reported that when these cells become attached to the capillary wall, as a first step toward emigration, they are buffeted by the swift current and frequently dislodged and whirled away. This does not happen in the venules, which provide ideal conditions for emigration.

Under ordinary conditions, with many capillaries closed down, there is still a current in the venules, and here the opportunity for exchange with the tissues cannot but be best. The view has been advanced that the rapid current of capillaries is especially favorable to gas exchange with the tissues, and the slow stream of the venules to that of less diffusible substances (18). However this may be, there is no doubt that the latter, when circulating transiently like our dyes, will get out in greatest proportion, other things being equal, where the current is slow. Whether circulating antibodies and the blood proteins reaching the lymph pass out preponderantly through the walls of the venules, present evidence does not enable us to say. Doubtless many factors condition their escape besides the immediate permeability of the vascular tube.

In man the skin of the body acts importantly to regulate temperature, as it does not in furred animals. Lewis has pointed out the obvious rôle of the broad, superficial venous plexuses in this relation. And in mammals generally the skin is one of the main depots of reserve water. There are important occasions, notably after hemorrhage, when water must be mobilized rapidly for circulatory purposes. The broad, highly permeable venular channels provide opportunities for this. In a preceding paper we have described vascular arrangements in the mammalian diaphragm and the pectoral muscle of the pigeon which are obviously adapted to the elimination of waste materials from these actively functioning organs (10).

It may be asked, why skin capillaries exist at all if venules do so well? The question has special pertinence in the case of human skin with its

great development of venules. But some pressure-regulating mechanism there must be; and the capillaries are known to share with the arteries in this function. They withstand pressure as the thin-walled venules cannot, because of their wide lumen. Diapedesis under pressure occurs first from the venules (16), and ecchymosis from these vessels is far more frequent than is generally recognized (19). Under circumstances of venous obstruction Tannenberg observed diapedesis taking place from the venules, then from the venous end of the capillaries, and only later from the arterial portion of these latter, despite the fact that through them the pressure was transmitted that was responsible for the lesions (17).

In preceding communications we have shown that in some regions an effective exchange with the tissues takes place through the small arterioles and venules, capillaries being few or absent where these run (1). From the point of view of exchange with the tissues no reason exists for a tripartite classification of the vessels of such regions. But in the skin the venules are not merely enlarged capillaries, functioning like them. Besides serving for exchange with the tissues and for the collection of blood they have other important purposes.

SUMMARY

The permeability of the venules of the skin of the mouse greatly exceeds that of the capillaries. A mounting gradient of permeability exists along the further portion of the latter.

The significance of these facts is discussed with relation to conditions in human skin. The cutaneous venules are differentiated for several functions besides those ordinarily attributed to them, and must be considered as specialized organs.

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EXPLANATION OF PLATES

PLATE 50

FIG. 1. Flap of the abdominal skin of a thin mouse, everted 6½ minutes after an injection of Chicago blue,—to show the dotting with color. The staining is palest along the midline. The unstained rectangular area near the upper right hand corner is the navel. $\times 8$.

FIG. 2. A similar flap everted 45 minutes after the dye injection,—to show the generalized staining. Some deeper dots of color can still be made out. $\times 8$.

FIG. 3. A part of the preparation used for Fig. 1, more highly magnified. The stain can be seen to lie in the tissue round about the venules. Here and there the arterial network can be perceived (A, A) but the arterioles are invisible. The interrupted white lines are ranks of hair follicles. $\times 17$.

FIG. 4. Under side of the abdominal skin of a mouse killed 7½ minutes after the dye injection. The venules are completely hidden in stained tissue. Enough dye remains in some of the arterioles (indicated by arrows) for their situation in unstained tissue to be made out. The contrast has been emphasized with a color filter. $\times 17$.

PLATE 51

FIG. 5. Skin of the abdomen of a mouse viewed from beneath. Specimen cleared after injection with an ink-gelatin mass. For comparison with Figs. 1, 3, and 4. From the venous and arterial networks (V and A) branches go off at intervals to the corium. Capillaries are not especially abundant in the neighborhood of the venules. $\times 75$.

FIG. 6. Section of the everted skin of a thin mouse, from the abdomen. The end, M, was near the midline. The specimen consists almost entirely of corium. There are no recognizable fat cells, and the panniculus carnosus, which is shallow, thins in the direction of the midline. Several hair follicles have been cut through. Eosin and methylene blue. $\times 180$.

FIG. 7. Section of the ear of a mouse near its margin. The upper side of the organ is distinguishable by the layer of fatty cells overlying the cartilaginous lamella. The gap to either side of the cartilage is an artefact. Hematoxylin and eosin. $\times 180$.

FIG. 8. A similar specimen with ink-gelatin mass in the vessels. Hematoxylin and picric acid. $\times 180$.

PLATE 52

FIG. 9. Ear of a living mouse photographed in oil 30 seconds after an intravenous injection of Chicago blue. As yet no dye has passed out into the tissues. The arteries, thin and straight, are readily distinguishable. Some of those which course separately are indicated by arrows. $\times 13$.

FIG. 10. The same ear photographed 5 minutes later. The solitary arteries can still be readily discerned (arrows). They pass to tissue which is unstained, whereas that in the neighborhood of the venules is already well colored. The dye has not escaped from the larger veins, however. $\times 13$.

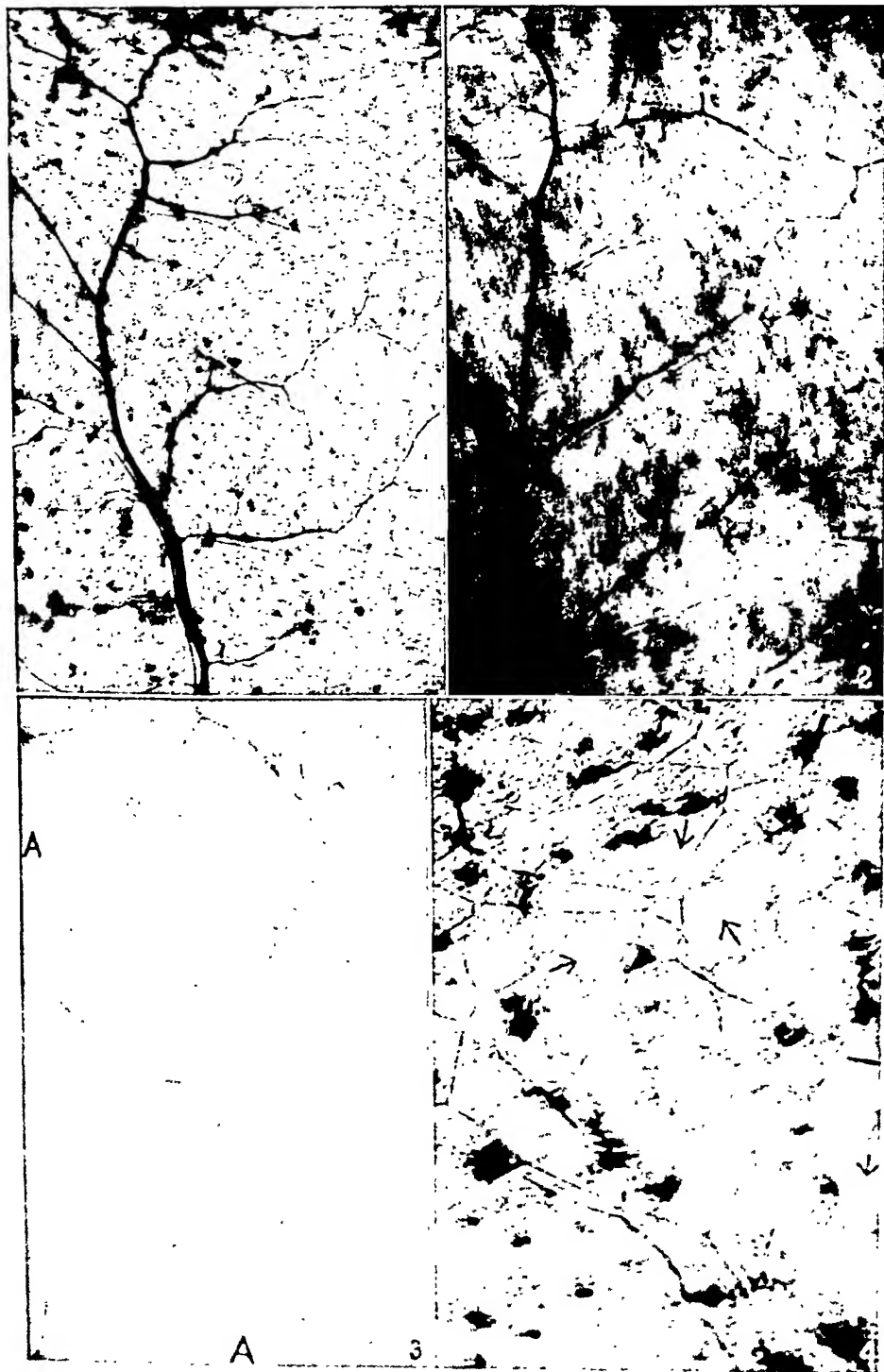
FIG. 11. A relatively high magnification of part of an ear cleared after injection with an ink-containing mass. Almost every artery is now seen to be accompanied by a vein. Several grids of the fine capillaries which supply muscle fibres can be made out near the lower border of the photograph (arrows). $\times 32$.

PLATE 53

FIG. 12. Mouse ear injected with an ink mass and just sufficiently cleared for the vessels of its outer side to be plainly visible. For comparison with the photographs from animals receiving vital dyes. The vascularization is much more abundant than one would suppose from the study of these latter. $\times 12$.

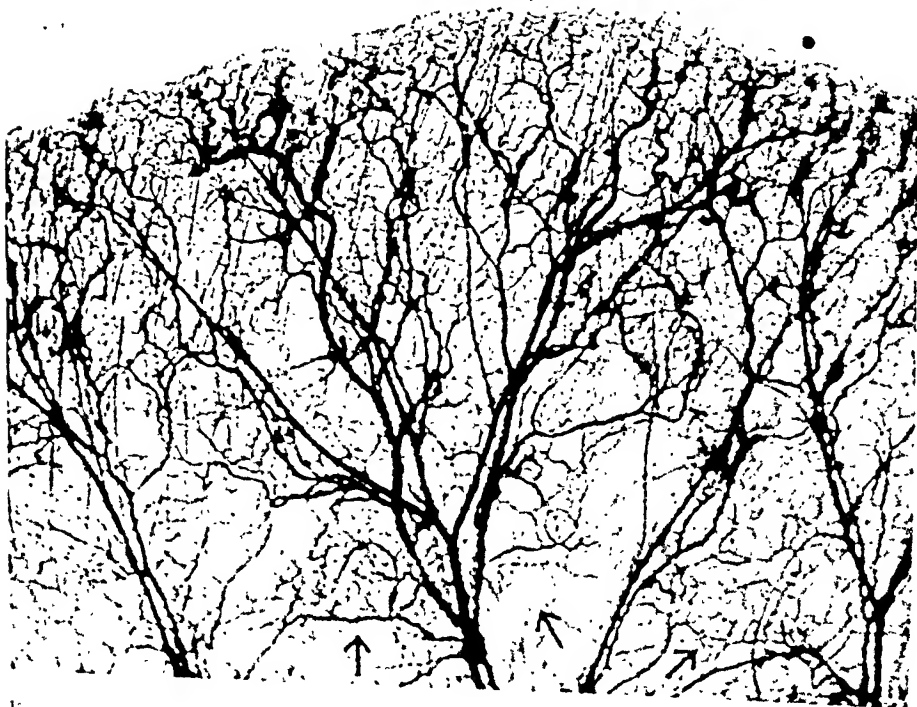
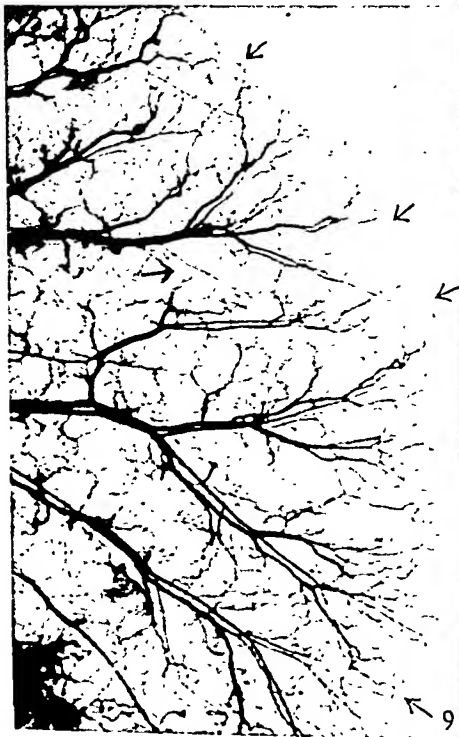
FIG. 13. Ear of a living mouse photographed 2 minutes after an injection of brom phenol blue. Some general staining has already occurred. It is least marked in the regions to which the solitary arterioles run (arrows). There are many broad patches of color with venules in their midst. $\times 13$.

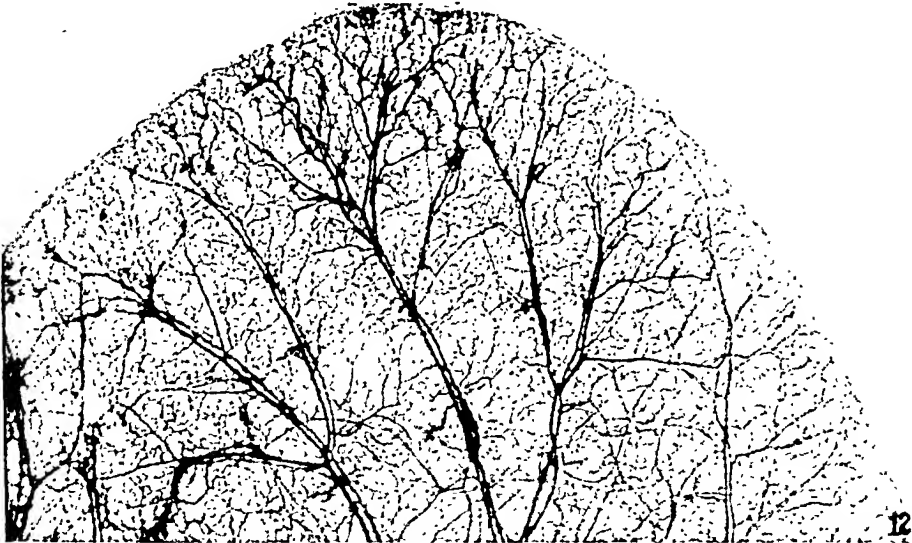
FIG. 14. Ear of a living mouse $3/4$ minute after injection of patent blue V. Some general staining has already taken place, which is not evident in the photograph. The blood of the venules contains so little dye that their course is difficult to make out. The solitary arteries on the other hand are clearly visible (arrows) by reason of their dye-laden contents; yet in the regions to which they run the staining is at a minimum. $\times 13$.











12



13



14

PROTEIN FRACTIONS OF A SCARLATINAL STRAIN OF *STREPTOCOCCUS HEMOLYTICUS**

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As a part of a series of investigations on streptococcal diseases now being carried on in this Department the writers have been attempting to separate and study immunologically active constituents of the scarlatinal type of *Streptococcus hemolyticus*. The work of Hitchcock (1) and the studies of Lancefield (2) have shown that as far as the microbial cell itself is concerned, the protein constituents are the dominating factors, and a study is accordingly being made of these.

The classical method for the isolation of the protein of the bacterial cell involved the use of sodium hydroxide in excess and made little attempt at fractionation (3). It has, however, long been recognized that alkaline solutions can induce profound modifications in native protein, and this is particularly true in the case of the nucleoproteins (4), the group to which the more easily isolated bacterial proteins are believed to belong. Thus Levene (5) extracted dried ground tubercle bacilli with 8 per cent ammonium chloride and found three nucleoproteins in the solution, while Eisler and Silberstein (6) used neutral saline extracts in studying typhoid nucleoproteins as precipitinogens. Also to avoid in part the effect of alkali Johnson (7) proposed to extract the ground, defatted cells first with water, reserving alkaline extractions of the residue for subsequent fractions. This method has been used in the Yale laboratory for the extraction of proteins from members of the tubercle bacillus group, and has been applied to Type III pneumococcus by Stull (8).

In the living microorganism the cell proteins, if not in actual solution, are at least largely present in the form of their salts with bases,

* The work reported in this communication was made possible by the Harkness Research Fund of the Presbyterian Hospital.

and this applies particularly to the acidic nucleoprotein group. Native and undenatured cell protein salts should therefore be largely water-soluble, hence the first extraction in the Johnson method might easily remove a mixture which would be difficult to separate.

The method used in the present study was intended to avoid this possibility and at the same time to test the idea that different cell proteins might vary in their acidic strength. It was felt that an effective method of separating such proteins would be to bring them all past their isoelectric points to a degree of acidity which would set them free from the base with which they were combined in the living protoplasm, and then to extract the acidified cellular material successively with buffer solutions of increasing basicity. Thus the strongest protein acid would dissolve in a buffer solution of comparatively low basicity, while a weakly acidic protein would require a more strongly alkaline buffer for its solution.

That the method may be of some utility is indicated by the separation from a scarlatinal strain of *Streptococcus hemolyticus* of a labile, alkali-sensitive, strongly acid nucleoprotein, differing distinctly in its physical and chemical properties, in its specificity, and in its reactivity toward human sera from the fractions obtained by subsequent extraction of the cell residue with more alkaline solutions.

The data given below are to some extent preliminary, but are reported in the hope that the method used might also be of value in the investigation of the proteins of microorganisms other than those being studied in this laboratory.¹

EXPERIMENTAL

1. Extraction and Isolation of the Acetic Acid Precipitable Protein Fractions.—Strain C₂O₃, a scarlatinal *Streptococcus hemolyticus*, obtained through the courtesy of Mrs. Lancefield, of the Hospital of The Rockefeller Institute for Medical Research, was used since it was reported to contain the type-specific factor. The culture was kept virulent by frequent passages through mice. 50 to 60 l. of a 3 day old culture in proteose-peptone broth were run through a jacketed, steam driven, sterilizable Sharples supercentrifuge designed by Dr. Franklin A. Stevens of this Department to prevent escape of spray. The microorganisms were scraped from the bowl, resuspended in about 400 cc. of saline, and centrifuged. From this point on, all operations were conducted in the cold with chilled reagents unless

¹ Studies on streptococci and the tubercle bacillus group are now in progress.

otherwise specified, the centrifugations, as well, being run in a refrigerating centrifuge (International Equipment Co.) provided with external cooling coils only. The washed cells were suspended in about 400 cc. of acetone, stirred frequently during 2 hours, centrifuged, taken up again in the same quantity of acetone, and allowed to stand overnight. After centrifugation the cells were taken up in 400 cc. of ether which had been freed from alcohol, peroxides, and aldehydes by washing with water, drying with calcium chloride, and storing in the dark over calcium chloride and sodium hydroxide pellets. After frequent stirring during several hours the mixture was centrifuged and the process repeated with a new lot of purified ether. A third lot was finally added and the mixture was allowed to stand overnight, after which it was centrifuged and the organisms were dried *in vacuo*. The yield was about 10 gm. under favorable conditions, about 10 per cent consisting of ash. The acetone-ether extracts were concentrated to dryness *in vacuo* and designated fraction A. The defatted cells were transferred to a ball mill at room temperature and rotated until intact cells could no longer be observed in a smear. The ground material was then rotated in the ball mill with a little 0.2 normal acetate buffer at pH 4 for 10 minutes and rinsed into 5 l. of the same buffer solution and stirred mechanically (all operations in the cold) for 5 to 6 hours, after which the mixture was run through a Sharples supercentrifuge. The effluent, clear after two passages through the machine, contained only 0.125 gm. of nitrogen in the one case in which this was determined, and reacted strongly for specific polysaccharide. It was designated fraction C, and the isolation of the polysaccharide from it will be described later.

The residue in the centrifuge bowl was ground in a mortar with (chilled) $M/15$ phosphate buffer at pH 6.5, added to 4 l. of the same solution, and stirred mechanically for 6 hours. The mixture was run through the supercentrifuge until clear, chilling as much as possible, and acidified with glacial acetic acid until the resulting precipitate began to flock (fraction D). The residue in the centrifuge bowl was ground in a mortar with water and extracted as before with 3 l. of water, adding enough normal ammonium hydroxide from time to time to maintain the pH at about 8.4, or just pink to phenolphthalein. The mixture was again cleared as well as possible and the effluent cautiously acidified with 50 per cent acetic acid until the precipitate began to flock (fraction E). The slimy residue in the bowl was then treated as before, carrying out the extraction at a pH at which phenolphthalein turned bright red (about 9). The precipitate yielded by the effluent on acidification with normal acetic acid was designated fraction F. The insoluble residue was then given a final extraction either at room temperature at pH 9 to 10, or in the cold at about pH 11 (blue to thymolphthalein), and the acetic acid precipitable material designated G. The generally small amount of insoluble cell residue (fraction N) was set aside for future investigation.

Fraction D was centrifuged off, leaving a clear supernatant at pH 3.8 to 3.9 (bromophenol blue). The Hastings-Sendroy color standard method (20) was used in determining pH in most cases. The precipitate was homogenized in about 200 cc. of water (chilled) and redissolved with the aid of chilled $M/15$ phosphate

sodium bicarbonate solution which had been neutralized to pH 6.8 by cooling and cautiously adding glacial acetic acid. Care was taken to leave the protein solution faintly acid to litmus at the end. After centrifuging, the small amount of insoluble material was discarded, and the protein solution was diluted to double the volume and reprecipitated with 50 per cent acetic acid. Centrifugation, solution, and reprecipitation were repeated four times, but before the final precipitation the solution was filtered through a 5 inch Berkefeld V candle which had previously been washed through with water containing a little sodium bicarbonate and finally with water. When necessary to produce a clear solution it was again filtered through a Berkefeld N candle. After flocculation of the protein in the above precipitations the supernatants were found to be in the range pH 3.8 to 4.0. The final supernatants and the final precipitate were free from inorganic phosphate. Fraction D was finally washed twice in the centrifuge bottle with water containing a little acetic acid, then twice with redistilled acetone, and was filtered off and dried *in vacuo* over calcium chloride, paraffin, and sodium hydroxide pellets. The yield in favorable cases was 1.4 to 1.5 gm. For analysis a portion was dried to constant weight in a high vacuum at room temperature over phosphorus pentoxide and sodium hydroxide.

Fractions E, F, and G were purified in the same way, using normal ammonium hydroxide for solution in the case of E, and sodium hydroxide in the case of F and G, and never allowing the solution to become more alkaline than about pH 8.5. As before, all operations were conducted in the cold. Supernatants from which E had flocked were usually at pH 4.0 to 4.1, those from F at pH 4.3 to 4.5 and those from G at 4.3 to 4.6. The yields of these fractions varied from 0.05 to 0.4 gm. each.

Each time E, F, and G were redissolved and the solutions centrifuged, particularly when the volume did not exceed 200 to 250 cc., brown, slimy material was deposited. This was collected and saved, and combined with the generally larger amount which collected on the Berkefeld V filter and often made filtration very slow. These slimy residues were ground in a mortar with water up to about 500 cc., made alkaline to about pH 9 (phenolphthalein), stirred thoroughly, and centrifuged for several days in the cold. The residue was treated again as before, until the maximum amount could be dissolved. The supernatants were acidified with acetic acid (to pH 4.6 to 4.8) and the precipitates combined and centrifuged off. Treated as before, very little alkali-insoluble material was deposited, and after a third reprecipitation the solution obtained on redissolving at pH 9 deposited almost nothing on centrifugation and could be run through a Berkefeld filter. The fraction which separated on acidification of the filtrate was designated L.

The supernatants from the original precipitations of C, E, F, and G, and the supernatants from the first two reprecipitations were concentrated to small bulk *in vacuo*, combined, dialyzed in collodion bags, and again concentrated *in vacuo*, made definitely acid with acetic acid, and centrifuged. The supernatant, fraction J, furnished additional specific carbohydrate, while the precipitate, fraction

TABLE I

Properties of Acetic Acid Precipitable Protein Fractions of Streptococcus scarlatinae

Fraction	$[\alpha]_D$	pH of solution used for $[\alpha]_D$ determination	N	P	Basic ash	N:P ratio
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
7 D	+9.5°	6.2	15.4	3.8	0.9	
F	-44°	7.4	14.9	1.4	0.3	
G	-49°	8.1	14.7	1.0	0.4	
L	-46°	8.4	14.4	1.4	0.3	
D ₁	-54°	7.6	15.8	1.2	0.1	
D ₂	-46°	6.5	15.1	2.6		
8 D	+12°	6.5	15.2	4.2	0.7	
E	0°	7.7	14.8	4.0	1.5	
F	-25°	9.0	14.2	2.1	0.4	
G	-55°	8.1	14.1	0.8		
H	+22°	7.0	15.8	5.2	1.0	
D ₁	-15°	8.2	15.5	3.2	0.5	
D ₂	-50°		15.0	1.5(?)		
9 D	+11°	7.9	16.0	3.8	1.1	
E	0°	6.8	14.5	2.8		
F	-42°		14.0	1.1	1.3	
D ₁	-64°	8.0	15.4	1.6	0.4	
D ₂	-34°	7.7	14.8	2.6		
15 D	0°	7.0	14.6	3.1	0.3	
E	-36°	8.3	15.4	1.9	0.5	
F	-44°	8.2	14.3	1.1	0.6	
G	-41°	7.2	14.0	1.1	1.2	
D ₁	-47°	7.8	15.0	1.6	0.0	
D ₂	-28°	7.4	16.3	3.3	0.6	
15 D ₁₂	+61°	7.1	15.9	8.9	0.9	1.78
D ₁₃	+19°	7.4	12.1	9.6		
7 D ₁₄	+16°		16.8	7.5		2.24
D ₁₅	+11°		15.4	8.5		1.81

H, was purified and isolated in the same manner as E, omitting filtration through a Berkefeld filter and reprecipitating only once.

Analytical data on the protein fractions are given in Table I. Nitrogen was determined by a slightly modified Pregl micro-Kjeldahl method, phosphorus by the Pregl-Lieb method, and basic ash by decomposing the sample on the water bath with a little nitric acid and igniting at a dull red with nitric and sulfuric acids. The ash was calculated as calcium, and $[\alpha]_D$, N, and P in the table were calculated on the ash-free basis, except in the few cases in which no ash determination could be made.

2. *Action of Dilute Alkali on Fraction D.*—0.5 gm. of 8 D was dissolved in about 35 cc. of water with the aid of 0.1 normal sodium hydroxide, added drop by drop until the solution remained just pink to phenolphthalein. 6.3 cc. were required. The volume was made up to 50 cc., and 150 cc. of water and 14.3 cc. of 0.1 normal sodium hydroxide were added. After 2 hours 66 cc. of water were added, so that, except for the first 2 hours, in which the concentration of alkali in excess was about $N/150$, the final concentration of alkali was 0.005 normal. The solution was allowed to stand at 23–26° for 24 hours and was then acidified with acetic acid until the precipitate flocked readily (pH 4.0). After standing overnight in the cold the mixture was centrifuged and the precipitate washed as in preceding examples with water containing a little acetic acid and with redistilled acetone. The amount recovered (D_1) was 0.35 gm. Nitrogen and phosphorus determinations on aliquot parts of the supernatant showed that 15 per cent of the nitrogen and 27 per cent of the phosphorus had been split off. A control portion of 7 D solution which had been diluted to the same relative volume without addition of excess alkali and reprecipitated, showed only 2 per cent of the nitrogen in the supernatant.

The actual amounts of nitrogen and phosphorus in the main supernatant were 11.06, and 5.55 mg., respectively, or approximately in the proportion to be expected if nucleic acid had been split off. The solution was concentrated to small volume yielding a small amount of precipitate on acidification with hydrochloric acid. $[\alpha]_D$ of the precipitate was approximately $+90^\circ$.

Since the degradation of fraction D in $N/150$ to $N/200$ alkali had caused only a reversal in the direction of optical rotation of the protein and a reduction of 1 per cent in the phosphorus content (see Table I), 0.056 gm. of D_1 was further degraded by dissolving as before in a few cubic centimeters of water and letting stand 24 hours with 0.02 normal sodium hydroxide up to 25 cc. On acidification with acetic acid 0.03 gm. (D_2) was recovered. The product now corresponded in its analytical data and optical rotation to the F and G fractions, except that the nitrogen content was higher. The value for phosphorus is based on a single determination and is probably too high, since duplicate analyses of the supernatant showed 73 per cent of the phosphorus (and 44 per cent of the nitrogen) to have been split off,—again in the ratio approximating that in nucleic acid.

A similar degradation of a mixture of 8 D and 9 D in 0.04 normal sodium hy-

dioxide at 25–30°C. resulted in a recovery at pH 4.3 of one-half of the protein as 9 D₁ with a somewhat greater negative rotation than 8 D₂, but otherwise showing similar analytical data. A small fraction, 9 D₂, containing more phosphorus, and evidently hydrolyzed to a smaller extent, was recovered on acidifying the supernatant from 9 D₁ with glacial acetic acid.

Attempts to determine whether or not nucleic acid was split from the protein were made as follows: 1.32 gm. of 15 D were dissolved as in the preceding cases, made up to 100 cc. with water and 20 cc. of 0.1 normal sodium hydroxide, and allowed to stand at 25° for 24 hours. The solution was alkaline to thymolphthalein and alizarine yellow R, but not to orange G or acid fuchsin, so that the pH was between 12 and 14. On acidification to pH 4.6, 0.77 gm. of D₁ fraction was recovered, and on adding acetic acid until the pH was below 3.6, 0.11 gm. of D₂ was obtained, D₁ and D₂ having the same significance as in the case of 9 D (see also Table I). The supernatant was concentrated to dryness *in vacuo*, taken up in a little water, freed from insoluble matter by centrifugation, chilled, and precipitated with 1:1 hydrochloric acid until the solution was acid to Congo red. The precipitate D_{3a} was collected separately and the supernatant treated with several volumes of alcohol, yielding a small amount of additional precipitate (D_{3b}). Each fraction was dissolved in a few cubic centimeters of water with the aid of normal sodium hydroxide solution, reprecipitated with hydrochloric acid, and washed with acidified water and redistilled acetone, using alcohol in addition for the reprecipitation and aqueous washings in the case of *b*. The yields were 0.13 gm. of *a* and 0.02 gm. of *b*. As will be seen from Table I the analytical data on the *a* fraction correspond with those for the nucleic acids (10).

A similar experiment with 7 D yielded analogous fractions, of which the D_{3a} fraction was much lower in optical rotation and somewhat higher in its N:P ratio than was the corresponding fraction from 15 D, the data in this case also being close to those given by the nucleic acids. Possibly it contained more of the levorotatory protein degradation product than did the nucleic acid from 15 D, although the biuret test was negative in a 1:1000 dilution, while it was faintly positive in a 1:200 solution of the 15 D product. When 2 mg. each of 7 D_{3a} and 15 D_{3a} were used for the orcin test, both gave typical pentose reactions, the final amyl alcohol solution showing an absorption band in the orange red.

3. *Occurrence of the Type-Specific Factor.*—Lancefield (2) was able, by means of acid extraction, to obtain from streptococci from widely different sources type-specific haptens which appeared to be protein degradation products. These were identified by their reaction with sera which had been absorbed by heterologous strains. Aqueous extracts of the organisms also contained type-specific material, but apparently not in antigenic form. Lancefield designated this the M factor.

It appeared of interest to locate, if possible, the type-specific material in the fractions prepared in the course of this work. Type-specific sera were obtained by Lancefield's method, with the aid of a vaccine prepared from the heterologous Strain S 24 of *Streptococcus hemolyticus*; the original culture of which was kindly furnished by Mrs. Lancefield. The results are given in Table IV, according to which fractions 8 H and 7 L contained the specific factor (Lancefield's M substance), in addition to non-specific material. That it possibly exists in antigenic form is indicated by the data on rabbit Serum 75 (Table IV). Two rabbits were injected intravenously three to four times a week for a period of 9 weeks with 0.6 to 1.5 cc. of a solution of 7 L containing 1 mg. per cubic centimeter. Each rabbit received a total of 33 mg. of protein. Bleedings 5 days after the last injection showed the presence of precipitins in the blood of Rabbit 75, and a fraction of these appeared to be type-specific antibodies since they were not absorbed by Strain S 24.

An attempt was also made to shorten the exposure of the streptococcus cells to acetone and ether, but in a case in which the washings were limited to a period of several hours very little material was obtained on extraction at pH 6.5 and its phosphorus content was only 2.7 per cent. Bacilli are known to contain nuclease (11), and it is possible the exposures to acetone and ether used in the case of Preparations 7, 8, and 9, while not too long to cause denaturation of the cell nucleoprotein, nevertheless sufficed to inactivate the nuclease presumably originally present. In Preparation 15 only the acetone extraction was shortened, but since in this case the first aqueous extraction at pH 4 was omitted and the D fraction was extracted with phosphate buffer at pH 7.1 it is uncertain to what cause to ascribe the slightly low rotation and phosphorus of the product and its undoubted contamination with fraction E. However, in Preparation 7 both of the first aqueous extractions were omitted and the initial one was made at pH 8.3. The possibility of simplifying the procedure with these points in mind is now under investigation.

DISCUSSION

It is shown in the description of the experiments that by a preliminary acidification of cautiously dehydrated, mechanically disintegrated scarlatinal *Streptococcus hemolyticus* and subsequent extraction in the cold with phosphate buffer at pH 6.5 it is possible to obtain a nucleoprotein radically different from that prepared according to the classi-

cal method involving alkaline extraction. The new nucleoprotein is characterized by its high phosphorus content, the low pH of its isoelectric point, its rotation of polarized light to the right, and its sensitiveness to alkali. Even in $N/200$ sodium hydroxide at room temperature the protein loses nucleic acid and becomes levorotatory, the hydrolysis or degradation product being poorer in phosphorus and isoelectric at a higher pH. In $N/50$ sodium hydroxide three-quarters of the phosphorus is eliminated and the nucleic acid set free may be isolated and identified. Since it shows a strongly positive pentose reaction it belongs to the yeast or pancreas nucleic acid type (10) rather than to that of thymus nucleic acid.

After removal of the nucleoprotein at pH 6.5 successive extraction of the cell residues at pH 8.4, 9, and 10 to 11 yields fractions characterized by their lower nitrogen percentage and poorer phosphorus content, particularly in the last two fractions. These fractions are also the most levorotatory. The three alkaline-extracted fractions appear to differ from each other chiefly in the progressive removal of a common phosphorus-containing component, presumably nucleic acid, with increasing strength of alkali used in the extraction, and differ little in physical properties and analytical constants, except for their lower nitrogen, from the protein fractions obtained by fractional degradation of the neutral-extracted nucleoprotein. An effort is being made to determine whether or not these two groups of fractions are identical.

Serologically the undegraded neutral-extracted nucleoprotein also differs radically from the fractions obtained by alkaline extraction after its removal, and from streptococcus nucleoprotein prepared by the classical method. Thus its reactivity is lower in the precipitin reaction in rabbit antisera obtained by intravenous injection of heat-killed or living bacilli, but it reacts more readily in many cases with the sera of patients suffering from different types of streptococcal disease (Table II). Moreover, the specificity of the neutral-extracted nucleoprotein is different from that of the alkaline-extracted fractions, since on absorption of rabbit antisera it does not remove the antibodies reactive against these fractions. On the other hand, absorption of the sera with the alkaline-extracted fractions removes the precipitins for the neutral-extracted nucleoprotein as well (Table III),

TABLE II
Precipitin Tests with Acetic Acid Precipitable Protein Fractions of *Streptococcus scarlatinae*

Fraction	Dilution	Rabbit antisera		Fraction dilution	Human sera				Antipneumococcus I, II horse serum
		R1289*	4 (1:1)		Ke (Nov.)†	Ke (Jan.)†	Ko (Dec.)†	Ko (Jan.)†	
7 D	1:20,000	+	—	8 D 1:1600 273§ 1:1600	(+++++)	(+) (—)			
F	1:20,000	++±	±						
G	1:20,000	++±	+						
L	1:5000	++±	++						
D ₁	1:1000		++						
D ₂	1:1000		++±						
8 D	1:5000	++	±†						
E	1:5000	++	±±						
F	1:5000	++	±±	9 F 1:1800	(—)	(—)			++±
G	1:5000	++	±±						
H	1:5000	++	±±						
D ₁	1:5000		+						
D ₂	1:5000		—						
9 D	1:1000		±†						
E	1:1000		+						
F	1:1000		±±						
D ₁	1:1000	R2485*	++						
D ₂	1:1000	++±	++						

Fraction	Dilution	R1:50*	Dilution of fraction	Serum 55	Fraction dilution	Human sera				Antipneumococcus I, II horse serum
						Fe	Br	M	T	
15 D	1:5000	++±	1:1000	+++	15 D 1:1000 1800	(++)	(+++)	(+±)	(++++±)	++±
E	1:5000	++±								
F	1:5000	++±								
G	1:5000	++±	1:1000	++++	15 G 1:1000	(+)	(+±)	(+±)	(+)	

Readings in parentheses determined after centrifuging at low speed.

* 1:1. Kindly supplied by Mrs. Lancefield.

‡ Data kindly furnished by Dr. Alvin F. Colburn and Miss Ruth Pauli of this Department.

† Turbidity, no whirl.

‡ Alkaline-extracted "nucleoprotein" corresponding to F or G. A 1:5000 dilution gave a +++ reaction in Serum 61, 1:1.

TABLE III

Absorption of Sera with Protein Fractions of Streptococcus scarlatinae

Test fractions at 1:1000 dilution. 0.3 cc. sera and fractions, 2 hours at 37°, overnight in the ice box, except as stated below.

Serum absorbed with	Test fraction											
	7 D		15 D		15 E		15 F		15 G		7 L	8 H
	±	-	+	±	±	+	+	+	+	+	±	+
7 D	±	-	±	±	±	+ <td>+<td>+<td>+<td>+<td>±</td><td>+</td></td></td></td></td>	+ <td>+<td>+<td>+<td>±</td><td>+</td></td></td></td>	+ <td>+<td>+<td>±</td><td>+</td></td></td>	+ <td>+<td>±</td><td>+</td></td>	+ <td>±</td> <td>+</td>	±	+
15 D	±	-	±	±	±	+ <td>+<td>+<td>+<td>+<td>±</td><td>+</td></td></td></td></td>	+ <td>+<td>+<td>+<td>±</td><td>+</td></td></td></td>	+ <td>+<td>+<td>±</td><td>+</td></td></td>	+ <td>+<td>±</td><td>+</td></td>	+ <td>±</td> <td>+</td>	±	+
15 E	±	-	±	±	±	+ <td>+<td>+<td>+<td>+<td>±</td><td>+</td></td></td></td></td>	+ <td>+<td>+<td>+<td>±</td><td>+</td></td></td></td>	+ <td>+<td>+<td>±</td><td>+</td></td></td>	+ <td>+<td>±</td><td>+</td></td>	+ <td>±</td> <td>+</td>	±	+
15 F	±	-	±	±	±	+ <td>+<td>+<td>+<td>+<td>±</td><td>+</td></td></td></td></td>	+ <td>+<td>+<td>+<td>±</td><td>+</td></td></td></td>	+ <td>+<td>+<td>±</td><td>+</td></td></td>	+ <td>+<td>±</td><td>+</td></td>	+ <td>±</td> <td>+</td>	±	+
15 G	±	-	±	±	±	+ <td>+<td>+<td>+<td>+<td>±</td><td>+</td></td></td></td></td>	+ <td>+<td>+<td>+<td>±</td><td>+</td></td></td></td>	+ <td>+<td>+<td>±</td><td>+</td></td></td>	+ <td>+<td>±</td><td>+</td></td>	+ <td>±</td> <td>+</td>	±	+

All controls,— except 15G,— saline, ±, ±, ±, ±.

All controls,— except 15G,— saline, ±, ±, ±.

First reading after 2 hours at 37°; second, overnight in the ice box; third reading after centrifugation at low speed. All

controls,—, except 15G—saline, ±, ±, ±.

Absorption of Serum 61 with Polysaccharide Fraction 8C

Serum absorbed with	Test fraction (1:20,000)			
	8 C	15 D	15 E	15 F
8 C	—	±	±	±
8 C + 15 D	—	± (Did not flock)	±	± (Flocked)

a behavior difficult to interpret until after these rather ill defined fractions (E, F, and G, the "nucleoproteins" as ordinarily obtained) have been subjected to more rigorous purification and study. Also, in agreement with Lancefield, the streptococcus proteins encountered in this study precipitate antipneumococcus serum (Table II).

Although absorption of rabbit antisera with specific polysaccharide isolated from fraction C failed to remove the protein antibodies or

TABLE IV

Absorption of Sera with Heterologous Strain S 24 of Streptococcus hemolyticus

Serum	Test fraction							
	9 D	8 D ₁	9 E	9 F	7 L	8 H	15 D	15 G
	Dilution							
	1:1000	1:1000	1:1000	1:2000	1:1600	1:1000	1:5000	1:5000
41 (1:2)		+±	++	+±				
41 absorbed (1:2)	-	-	-	-	+	+		
41 (1:1)						++±		
75 absorbed (1:2)					± (+)*	± (+)	- (-)	- (-)
	Dilution							
				1:2000	1:2000	1:2000		
41 (1:2) after 3 mos.				- (±)	- (-)	± (+)		
R _{1a} (1:1)					+±			

* Values in parentheses obtained after centrifuging at low speed.

alter the differences between them (Table III), in only one instance (8 D) was it possible to absorb with a protein fraction and leave polysaccharide antibodies in the serum.

It is of interest to note that the water-soluble protein fraction obtained by Stull from Type III pneumococcus (S) resembles in its nitrogen and phosphorus content the D fractions summarized in Table I, while the carbonate-extracted fraction is much lower in phosphorus. It is to be regretted that a serological study of these pneumococcus fractions was not reported.

With the aid of type-specific sera prepared according to Lancefield (2) the scarlatinal type-specific factor (Lancefield's M) was found in two fractions, and of these the one which was tested gave indications of containing the material in antigenic form. However, a solution of this fraction, 7 L, 3 months old, appeared to have lost its specific

TABLE V
Degradation of Fraction D by Sodium Hydroxide

Preparation	Concentration NaOH (normality)	Temperature	Time	Total N	Total P	Ratio $\frac{\text{Wt. N}}{\text{Wt. P}}$ split off
				In supernatant after precipitation with acetic acid		
		°C.	hrs.	per cent	per cent	
7 D	0		—	2		2.0
	0.25	24.5-25	24	77, 78		
	0.1	24.5-25	24	66, 63		
	0.05		24	59		
	0.01	24.5-25	24	42		
8 D	0.01	24	7	13	22	
	0.01	27-29	24	34		
	0.005	27-29	24	23		
	0.005	23-26	24	15	27	
D _I *	0.02	25	24	44	73	
15 D	0		—	4		
			min.			
	0.1	25	4	17		
			hrs.			
	0.1	25	1	41		
	0.25	37	48		No inorganic P (12)	

* Product of the treatment with 0.005 N NaOH.

reactivity, although the non-specific protein in the fraction seemed unaltered (Table IV). On the other hand, 8 H, kept in a desiccator and dissolved only as needed, has maintained its type specificity for a year.

It is evident from the last experiment reported in Table V that the D fraction cannot be classified as a phosphoprotein in spite of its high

phosphorus content, since inorganic phosphorus is not split off on treatment with 1 per cent alkali (12). Similar tests on an E and an F fraction also gave negative results. Since, however, the phosphorus is split off as nucleic acid from D the new protein belongs definitely to the nucleoprotein group. To consider it as a native cell protein on account of its great lability would be tempting, but premature, for on account of its high phosphorus content it is conceivable that scission from a portion of the originally combined protein had already occurred in spite of the many precautions observed to minimize enzymatic (13) or manipulative hydrolysis. Should the D fraction actually be a degradation product of the original protoplasmic nucleoprotein it would be necessary to classify it as a nuclein, a group of substances ordinarily obtained by peptic digestion of nucleoproteins.

Interesting from the standpoint of nucleoprotein chemistry is the fact that the new protein is not merely a salt of nucleic acid, but contains the nucleic acid in chemical combination. It yields no precipitate with one-third volume of 20 per cent barium acetate solution at pH 6.6 (14), nor are significant amounts of nucleic acid split off until the solution of the protein is rendered alkaline, after which the amount set free depends on the strength of alkali and the period of exposure (Table V). In this streptococcus nucleoprotein, then, the nucleic acid appears to be combined in a labile, ester-like linkage, slowly saponifiable even by alkali as weak as $N/200$.

The relation of the present work to Lancefield's studies (2) will be clearer when animals have been immunized to the various fractions and the properties of the antisera studied. Until then it may be said that the polysaccharide in fraction C is undoubtedly that designated as "C substance" by Lancefield, while the type-specific factor in fractions 7 L and 8 H is the same as Lancefield's "M substance," except that it appears to be a true antigen instead of a haptén. Fractions E, F, and G would correspond to Lancefield's nucleoprotein P. It is possible that the labile nucleoprotein, fraction D, corresponds to Lancefield's Y, present only in minor amounts in her preparations owing to the use of alkali in extracting the nucleoproteins and the use of hot acid in extracting the type-specific haptén. Should this surmise prove to be correct, Lancefield's Y factor would become known as one of the major antigens of the streptococcus cell.

So far in the present work the isolation of any protein not precipitable by acetic acid has not been attempted, but with the growth of larger amounts of organisms such proteins will be sought for and the study continued of the products already isolated.

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SUMMARY

1. A tentative method is described for extracting a labile nucleoprotein from scarlatinal *Streptococcus hemolyticus*.

2. The product differs chemically and serologically from the fractions prepared by subsequent alkaline extraction of the cell residues, and from protein obtained by the classical method for extraction of bacterial "nucleoproteins."

3. The new nucleoprotein is sensitive to very weak alkalis and readily loses nucleic acid under these conditions. The protein degradation products resemble the alkaline-extracted protein fractions of the cell residues.

4. The bearing of the properties of the new nucleoprotein on the chemistry of nucleoproteins in general is discussed, also the possible relation of the fractions obtained to the analysis of streptococcus antigens made by Lancefield.

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THE USE OF MICE IN TESTS OF IMMUNITY AGAINST YELLOW FEVER*

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The Need for a Test of Immunity against Yellow Fever

The study of the epidemiology of yellow fever would be greatly facilitated by some simple immunity test which could be applied to human beings on a large scale. With such a test it would be possible to determine whether yellow fever has been present in a region within the life of the present generation and to estimate the time and extent of the infection. By testing an adequate number of persons in selected places, it should be possible to map large areas with regard to the endemicity, epidemicity, or absence of yellow fever, and to decide where control measures are necessary. The test would be especially valuable where the disease is present but is seldom or never recognized, and also where there is confusion of yellow fever with other diseases, particularly leptospiral jaundice. Such a test is needed also for determining whether persons vaccinated against yellow fever by any of the proposed methods have in fact been immunized. Just at present there is a pressing demand for immunity tests in connection with yellow fever surveys of West Africa, whence aviation and other improved means of travel threaten to carry yellow fever past the little understood natural barriers to large susceptible populations in East Africa and the Orient. Numerous immunity tests will be needed also for delimiting the endemic areas of Brazil.

A very simple method of ascertaining whether a person is immune to yellow fever would be by a skin test, if a suitable antigen could be

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found, but Frobisher (1) was unable to produce significant skin reactions in immune monkeys by the intradermal injection of any of several antigens. The methods of testing sera for their power to fix complement in the presence of yellow fever antigens, as developed by Frobisher (2) and Gordon Davis (3), seem to give specific results, but fixation does not take place as a rule in the tests of the sera of animals which have been immunized by vaccination without the production of fever, or in the sera of some of the highly immune persons who have recently had yellow fever (Hudson (4)). While the method gives promise, it will not be ready for wide application, except as a supplementary test, until its limitations and methods of interpretation have been more exactly determined. The protection test in *Macacus rhesus* monkeys, on the other hand, has proved reliable and highly useful, but it cannot be applied on a scale large enough for present needs because too great a number of monkeys would be required. By testing human sera for their power to protect monkeys against yellow fever virus, Beeuwkes, Bauer, and Mahaffy (5) were able to determine the endemicity of yellow fever in parts of West Africa where cases were seldom recognized, and to show that certain other regions were free of the infection. By this same test the identity of the recent isolated outbreak of yellow fever in the interior of Colombia, described by Peña Chavarría, Serpa, and Bevier (6), was confirmed in the laboratory in New York by Hudson and Kitchen (7).

The protection test has many advantages and would meet the needs of the investigators in the field if a larger number of sera could be examined than is possible when monkeys are used. Any one of the potent African strains of yellow fever virus could be used in testing sera from any part of the world, for the serological identity of the African and American strains has been amply demonstrated by cross-immunity tests (Sawyer, Kitchen, Frobisher, and Lloyd (8)) and by the growing experience with protection tests in which sera from many parts of the Americas have protected against African strains of virus. The protection test can, as a rule, be depended upon to reveal immunity resulting from attacks of yellow fever no matter how long past. For example, specimens of serum from six persons who were ill during the epidemic in Norfolk, Virginia, in 1855 were obtained 75 years later, and five of the six sera protected monkeys against African yellow fever

virus (Sawyer (9)). Protection tests of the sera of a sufficient number of persons classified by location and age group would permit the reconstruction of at least the recent yellow fever history of a region and would thus help in solving the present problem of control.

The Susceptibility of the Mouse to Yellow Fever

When the susceptibility of the white mouse to the virus of yellow fever was announced in 1930 by Theiler (10, 11), it seemed that the laboratory animal needed for replacing the monkey in the protection test had been found. Working with the French strain of yellow fever virus from Africa, he discovered that adult mice could be infected by intracerebral inoculation but not ordinarily by subcutaneous or intraperitoneal injections.

Theiler found that the virus was highly neurotropic in mice and was abundant in the brain tissue after the disease had developed, although absent from the blood or liver. There was a heightening of the virulence for mice with successive passages in these animals, until a fixed strain for mice was produced which caused the death of all the animals inoculated intracerebrally in approximately 5 days after injection. Theiler showed that experimental yellow fever in mice took the form of an encephalitis with characteristic changes in the nuclei of the ganglion cells. Outside the nervous system the only lesion found which might be characteristic of yellow fever was hemorrhage into the stomach and intestines, but this occurred only in some of the infected animals. He showed also that the sera of immune monkeys or persons had the power to prevent or defer the disease if mixed with the virus before the intracerebral injection. Variation of the length of time that the virus and serum were in contact before injection seemed to make no difference in the degree of protection. Injections of large amounts of immune serum intraperitoneally seldom protected against virus inoculated intracerebrally.

The Protection Test by Intracerebral Inoculation of Mice

In a later publication Theiler (12) has presented experiments with a method for testing sera for their protective power against the fixed virus for mice. He added each immune serum to an equal volume of a suspension of the virus, and inoculated mice intracerebrally with the mixture, using six or more mice in each test. Control groups of mice received normal serum in the place of the immune serum. Nearly all the control animals died from 5 to 9 days after inoculation. Of the mice which had received immune serum, usually a few died like the control animals, a few more died from the 10th to the 16th day, and several survived. Even when the virus suspension contained only 1 per cent of virus and had been centrifuged to remove all particles, a considerable proportion of the animals which received immune serum and virus died within the period of observation. The test had also

permitted differentiation between immune and non-immune sera, but the irregularity of the results would make it necessary to use an excessive number of animals to reach valid conclusions in testing large numbers of specimens. If this test can be made more highly reliable by quantitative adjustments, it will have the advantage over the modified test which we shall describe of requiring only very small quantities of serum and virus.

Establishment of New Strains of Yellow Fever Virus in Mice

In our study, the susceptibility of the white mouse to yellow fever virus when inoculated intracerebrally was confirmed.

The Asibi strain of yellow fever virus of African source was established in mice by our associate in the laboratory, S. F. Kitchen, and we likewise established the S. R. strain from Brazil and the P. A. L. strain. Theiler kindly let us have the French strain in its 95th passage in mice, and most of our work was done with this strain, which has now reached its 141st passage. The Asibi strain has been carried through 51 passages, the S.R. through 21, and the P.A.L. through 15. These new strains in mice have gradually become more like the fixed French strain, but only the Asibi strain has reached a point at which it may be considered as fixed. In Table I are shown the intervals in days between the intracerebral inoculation of mice with each of these strains and the time when the animals were found dead or were killed when definitely ill. The periods shown give a close approximation to the incubation periods, as the time interval between the observance of definite symptoms of weakness or partial paralysis and the death of the animal is usually short, often only a few hours but not infrequently 1 day. Many of the mice inoculated with the French strain in the latest series in the table were killed so that their brains could be used for inoculation. If they had not been killed, a few of them would doubtless have died during the following night and their deaths would have been recorded as occurring 1 day later. The differences shown in the table between the results with the earlier and the later passages of the French and Asibi strains of virus are of doubtful significance. The virus was already fixed in the earliest passages available for the comparison. It is obvious, however, that both these fixed strains produce death in a shorter time than do the two strains which have been passed through mice only a small number of times. The mortality is not shown in the table, as nearly all the mice died that were used in the experiments on which the table is based.

Observations made before Nov., 1930, are excluded from Table I, as many of our earlier experiments were made with a strain of mice of low susceptibility to yellow fever virus, and the results were therefore not comparable with those in the table.

With Kitchen we have fully confirmed Theiler's observations (10) that yellow fever virus, after a number of passages in mice, will still

produce experimental yellow fever with typical lesions in *rhesus* monkeys, but that the virus finally loses its power to produce fatal attacks in monkeys after many passages in mice. We shall reserve details of these observations for a later paper by Kitchen and ourselves dealing with vaccination against yellow fever. The pathology of experimental yellow fever in mice is being studied by Kitchen and will be described by him in a later publication.

Protection Test by Intraperitoneal Inoculation of Mice

In applying the protection test by intracerebral inoculation, we obtained results similar to those of Theiler, and came to the conclusion

TABLE I

Effect of Numerous Passages of Yellow Fever Virus of Several Strains on the Time Interval between Intracerebral Inoculation and Death

Strain of yellow fever virus	No. of passages in mice	No. of animals dying, or killed when ill, after each of the following intervals in days after inoculation									
		4	5	6	7	8	9	10	11	12 to 14	
French	Earlier: 105, 106, 107	3	35	21							
	Latest: 138, 139, 140	133	276	7	2						
Asibi	Earlier: 32, 33, 34	5	17	4	4						
	Latest: 48, 50, 51	3	8	14	4						
S. R.	Early: 3, 4,				6	5	4	1	1		
	Latest: 19, 20, 21		4	11	6	2					
P. A. L.	Early: 5, 6, 7	1	4	13	12	10					
	Latest: 13, 14, 15		3	7	7	5					

that we could not expect the small amount of immune serum injected intracerebrally to protect completely and regularly against fixed yellow fever virus placed in direct contact with injured brain cells. We therefore sought some method by which more serum could be injected and the virus could be brought less directly and more slowly to the brain.

It seemed probable that massive intraperitoneal injections of the virus in brain tissue would cause the introduced virus to circulate in the blood for several days, for Sawyer and Probstner (13) had shown

that yellow fever virus could be recovered for 2 or more days from the heart blood of several resistant animals inoculated in this way (guinea pig, ferret, bullfrog). This circulation of virus was evidently not necessarily related to any infection either inapparent or obvious which might develop later, because it occurred in the entirely insusceptible bullfrog. To enable the circulating virus to attack the brain of the adult mouse it might be necessary to cause some mild injury of the brain tissues, because simple intraperitoneal inoculation alone does not produce encephalitis in the adult animals. If infection could be brought about in this way, it should be possible to test sera for protective power by injecting them intraperitoneally in adequate amounts together with the virus and at the same time producing a mild injury to the brain.

There were precedents for the application of the principles involved in the method proposed. Flexner and Amoss (14) reported in 1917 that "normal monkey or horse serum, isotonic salt solution, and Ringer's and Locke's solutions, when injected into the meninges, promote infection with the virus of poliomyelitis introduced into the blood, the nose, or the subcutaneous tissues." Zwick, Seifried, and Witte (15) found out that the cutaneous inoculation of rabbits with Borna virus succeeds only in exceptional instances, but that infection is easily brought about by such inoculation if at the same time an injury is produced in the central nervous system by the injection of non-specific fluids (normal rabbit serum, normal horse serum, or isotonic sodium chloride solution).

The Agent for Localizing the Virus in the Brain

In preliminary trials many fluids were injected intracerebrally into mice and compared for their effect in localizing yellow fever virus in the brain.

The virus was injected intraperitoneally, as 0.2 cc. of a 10 per cent suspension of infected mouse brain, immediately after the anesthetized mouse had been given the intracerebral injection. The substances tested as the intracerebral inoculum were distilled water, 0.9 per cent and 3 per cent sodium chloride solutions, normal human and normal horse sera, solutions of magnesium chloride, magnesium sulfate, potassium alum, and manganese chloride, 10 per cent extract of mouse testicle in 0.9 per cent sodium chloride solution, and solutions of corn-starch, tapioca, and wheat flour. Simple needling of one or both sides of the brain was tried also. Some of the salts were too toxic in the strengths of the solutions used and were abandoned. Others of the solutions seemed to have uncertain or inadequate effects.

Satisfactory results were obtained with each of the three solutions of starch in either distilled water or 0.9 per cent sodium chloride solution. The 0.5 per cent magnesium chloride solution seemed almost equally effective and was used in a considerable number of the earlier tests. Our final choice of the fluid for injection in routine tests was a 2 per cent solution of starch in 0.9 per cent sodium chloride solution injected intracerebrally in the amount of 0.03 cc., and this solution has been used in all the more recent tests.

At the time of these early comparative tests we were using strains of mice of low and varying susceptibility and, as a result, an accurate comparison was impossible. Working more recently with highly susceptible mice, we repeated many of these experiments and found that most of the mice succumbed to yellow fever encephalitis regardless of which substance was injected, and even when the conditions were made less favorable by a reduction in the amount of virus inoculated intraperitoneally. Simply inserting the hypodermic needle into the brain often proved sufficient to establish the infection. The results of these recent tests are given in Table II. While these tests would seem to allow considerable latitude in the choice of the intracerebral inoculum, we have decided to continue using the 2 per cent starch in isotonic sodium chloride solution. It seemed to us probable that the slower absorption of the starch and the presumably more definite injury to the tissues would make it more reliable than simple needling or the injection of isotonic salt solution alone. In any event the starch solution is proving effective in our experience with the protection tests in mice.

The Intraperitoneal Inoculum

In the production of experimental yellow fever in mice by the simultaneous injection of virus into the peritoneal cavity and a mildly injurious substance into the brain, we found that a considerable proportion of the animals escaped infection unless the amount of virus injected was large.

In one comparative test the brain injury was produced by the injection of starch solution and the intraperitoneal injection of virus consisted of a 10 per cent suspension of the brain of an infected mouse in isotonic salt solution. An equal volume of normal human serum was injected with the virus. When the amount

TABLE II
*Comparative Tests of Mechanical Injury and the Injection of Various Fluids for
 Their Power to Localize Yellow Fever Virus in the Brain of the Mouse*

Fluid injected into brain to localize the virus (0.03 cc.)	Percentage of infected mouse brain in intra-peritoneal inoculum (0.2 cc.)*	No. of mice inoculated and alive 4 days later	No. dying from 5 to 10 days after inoculation	Per cent dying within this period
No fluid (only insertion of needle)	20 5 1	12 12 11	10 7 8	83 58 73
0.9 per cent sodium chloride	20 5 1	12 12 12	10 11 6	83 92 50
3 per cent sodium chloride	20 5 1	6 11 11	3 8 4	50 73 36
0.5 per cent magnesium chloride†	20 5 1	12 11 10	10 11 6	83 100 60
Normal horse serum	20 5 1	12 11 9	10 6 6	83 55 67
20 per cent extract of mouse testicle†	20 5 1	12 11 10	10 10 5	83 91 50
2 per cent starch solution†	20 5 1	12 12 12	10 9 7	83 75 58
Combined results with all of above injurious agents	20 5 1	78 80 75	63 62 42	81 78 56

* Virus: French strain 139th and 140th passages in mice. Tests with the 20 per cent suspension were made together as one experiment, and the other tests comprised a second experiment. Each mouse received 0.4 cc. of normal human serum in the first experiment and 0.4 cc. of a 10 per cent solution of normal human serum in 0.9 per cent sodium chloride solution in the second.

† Dissolved or suspended in 0.9 per cent sodium chloride solution.

of the brain suspension injected was 0.5 cc., all of ten mice inoculated died, but when the amount was 0.1 cc., only three of five mice died. In another experiment the concentration of the virus suspension was varied while the volume was kept constant at 0.2 cc. Normal human serum (0.4 cc.) was injected with the virus into each animal. The intracerebral injection consisted of 0.03 cc. of 2 per cent starch.

As is shown in Table III, the animals receiving virus in brain suspensions of a concentration of 10 per cent, or over, all succumbed, while a considerable proportion of those receiving suspensions of 1 per cent or less survived. Likewise in the experiments reported in

TABLE III

Variation of the Mortality Rate of Mice with the Amount of Virus in the Intraperitoneal Inoculum

Mouse brain in virus suspension*	No. of mice inoculated and alive 4 days later	No. of these mice dying 5 to 10 days after inoculation	Mice dying
<i>per cent</i>			<i>per cent</i>
40	4	4	100
20	5	5	100
10	11	11	100
1	5	3	60
0.1	8	1	13
0.01	8	1	13

* Virus: French strain, 111th passage in mice. Volume of the virus suspension injected intraperitoneally, 0.2 cc. With it was mixed 0.4 cc. of normal human serum. Simultaneous intracerebral inoculum: 0.03 cc. of 2 per cent starch solution.

Table II the number of fatal infections was much smaller when the intraperitoneal inoculum was a 1 per cent suspension of virus than when it was a 5 per cent suspension of the same virus.

Evidently the chance of producing encephalitis in all the mice of a group inoculated by the method used is greater when the larger quantities of virus are injected. There are practical difficulties, however, without compensatory advantages in using very large amounts.

The observations in Table II show that the use of even as much as 0.2 cc. of a 20 per cent suspension of brain virus may allow some of the animals to survive, and the experience of the laboratory, in testing several hundred sera, was that

this amount of virus would often permit one mouse to survive, and sometimes two in a group of six control animals receiving the virus with normal human serum.

To secure the necessary amount of 20 per cent virus suspension for the test approximately as many mouse brains were required as there were specimens to be tested, and this made necessary the inoculation of many animals as a source of virus. As the substitution of 10 per cent virus for 20 per cent made only a small difference in the accuracy of the test, when highly susceptible animals were used and resulted in a great saving of animals, we have recently adopted 0.2 cc. of 10 per cent mouse brain virus as the virus component of the intraperitoneal inoculum and have paid special attention to the securing of suitable mice.

Isotonic sodium chloride is the fluid in which the brain was at first suspended in the preparation of the virus for inoculation, and it is satisfactory if the virus is freshly prepared just before it is mixed with the undiluted sera to be tested. In the light of the experience of Bauer and Mahaffy (16), who found that the addition of 10 per cent of normal monkey serum to diluted virus suspensions helped preserve the virus, we prefer to add this amount of a normal serum (human or monkey) to the salt solution used as a diluent in the test.

We have found that 0.4 cc. of the serum is a satisfactory amount for the test. When this quantity is mixed with the virus suspension and injected, the test is so sensitive that sera from yellow fever convalescents diluted to 1 per cent usually protect a considerable proportion of the mice.

Technic of the Test

Specimens to be Examined.—At least 6 cc. of the serum to be tested is needed. The test requires 3 cc. The remainder is used in case the results of the first test are doubtful or the control tests prove unsatisfactory.

The Mice.—Healthy young adult white mice of about 20 gm. weight are preferred, but moderate differences in age do not seem to affect the results of the test. A pure strain of mice, tested and found highly susceptible and bred on the premises would give the best results. Satisfactory work may be done with susceptible mice purchased from dealers, but each supply from a new source should be tested for susceptibility and there should be an understanding with the dealer that he will not change the source of his mice without notifying the laboratory. During the test mice may be fed exclusively on bread soaked in milk, given once each day, and they will then require no water. During the experiments the mice are kept in glass battery jars with wire mesh covers, six mice in each jar. Wood shavings are placed in the jars, and the mice are moved to fresh jars 1 week after inoculation.

Preparation of the Virus.—An approximate time is set in advance for the tests preferably 2 or 3 consecutive days in the middle of the week. 5 days before each testing date, a sufficient number of healthy mice are inoculated intracerebrally with yellow fever virus in mouse brain tissue. The mice are inspected each morning. The definitely sick animals are killed with chloroform and are pinned out back down. A rapid and simple necropsy is performed to find out if there are gross

lesions of any disease other than yellow fever and to drain the blood from the brain. The only gross lesion probably due to yellow fever, which has been observed, is hemorrhage into the stomach and intestines, and this is absent in most cases. The mouse is then turned over and pinned out. The skin is slit with scissors from the nose to the middle of the back and the flaps are laid back. An assistant sears the top of the skull with a red-hot soldering iron. The operator removes the bony covering over the brain with sterile sharp-pointed scissors and then spoons out the brain with a rigid, narrow spatula of nickel. The brain is placed in a small, weighed petri dish. The other brains secured are placed in the same dish and the total weight is ascertained. The brains are then finely ground up in a porcelain mortar with enough isotonic salt solution, or preferably salt solution containing 10 per cent of normal serum, to make a 10 per cent suspension.

Preparation of the Serum-Virus Mixtures.—In advance of the tests, 3 cc. of each specimen of serum or diluted serum and of each of several sera for the controls is placed in a small test-tube. If less than 3 cc. of serum is available for a test, a 50 per cent solution of the serum may be tested if the dilution is stated in the report. If less than 1.5 cc. is received, we discard the specimen unless its unusual importance justifies testing it in higher dilutions. To each tube is added 1.5 cc. of the virus suspension. The contents of each tube are mixed and drawn up into a 5 cc. graduated glass syringe with the number of the specimen or that of the mouse group to be inoculated written on it with a wax pencil.

The Starch Solution.—The starch solution for intracerebral injection is prepared in advance by adding 2 per cent of corn-starch, such as is sold for food, to the 0.9 per cent sodium chloride solution and heating in a flask in a bath of boiling water. The solution is then placed in small wide mouthed Erlenmeyer flasks, autoclaved, and stored ready for use. In the morning of the day of the tests the starch solution is drawn up into tuberculin syringes of 0.25 cc. capacity, fitted with hypodermic needles 0.42 mm. in diameter (Stubs gauge No. 27) and 10 mm. long. The number of syringes filled is the same as the number of mouse groups to be inoculated.

Controls.—With each set of tests there should be five control groups of six mice each. Two groups should receive 0.4 cc. of normal serum (human or monkey) in place of the unknown serum of the test, and two should receive a known immune serum (human or monkey). To conserve the immune serum, it may be diluted to 10 per cent if of sufficiently high titer. The fifth control should be given the virus mixture intracerebrally. It shows whether the mice are susceptible and the virus potent. Intracerebral inoculation brings the animals down 1 day sooner than intraperitoneal inoculation with simultaneous cerebral injury.

An important object of the immune serum control is to show that the deaths caused by the virus suspension injected are in reality due to yellow fever and not to some contaminating organisms in the virus mixture. In our recent experience the most frequent cause of an unsatisfactory result requiring repetition of the tests is contamination of the virus as revealed by deaths of mice in the immune serum control groups. The organism of mouse typhoid, Strain 1, was isolated from the brain of one of the animals dying in an unsatisfactory test of this kind. We are

attempting to diminish this difficulty by using healthy mice of our pure strain bred on the premises for source of virus and inoculating them from mixtures containing the smallest possible number of brains. When it seems advisable as an additional precaution, we filter the brain suspension used in inoculating the source animals through Berkefeld N filters. It would be possible also to go back to stored, dried specimens of the virus which were obtained when it was apparently free from contamination. Before filtering the virus, the brain suspension should be centrifuged and the sediment discarded. The mouse brain virus may be easily preserved by preparing a suspension of the brain tissue in normal human or monkey serum, placing it in 1 cc. amounts in test-tubes, drying it in the frozen state, and then sealing the tubes and storing in the refrigerator. We have already preserved the virus in this way for several months. The essentials of the method have been published by Sawyer, Lloyd, and Kitchen (17).

Inoculation of the Mice.—An assistant anesthetizes the mice by placing several in a battery jar, on the bottom of which is a layer of cotton moistened with ether. The mice are taken from the jar when they become unconscious and are laid on the table near the operator. The operator, wearing rubber gloves, takes a mouse, lays it back up on a towel on the table, parts the hair of the head with a swab wet with 70 per cent alcohol and thrusts the fine needle of the tuberculin syringe through the thin skull and into the center of the brain. He injects 0.03 cc. of the starch solution. The mouse is then picked up by the skin of the neck, and its tail is held by the third finger of the same hand. With one of the larger syringes an intraperitoneal injection of 0.6 cc. of the virus-serum mixture is given. The mouse is then dropped into a numbered jar. One operator with an assistant can test up to 25 sera on each of 3 days of the week if he has an abundance of equipment ready in advance. The actual injections require approximately 3 minutes for each mouse group.

Inspection and Recording.—The mice are inspected every morning for 14 days, and a record is made of those that are sick or dead. Our printed record form on cards measuring 77 by 128 mm. is shown in Fig. 1. The first mouse to become sick or die becomes Mouse 1 of the group, and the second, No. 2, and so forth.

Interpretation of the Results.—The result of the protection test is recorded as the ratio of the number of mice surviving on the 10th day after inoculation to those that were alive and well on the 4th day, and this is followed in parenthesis by the corresponding ratio for the control groups receiving normal serum with virus, thus: "5/6 (0/12)," meaning that 5 out of 6 mice survived in the test group and none of 12 in the normal serum control groups. Deaths before the 5th day are in all probability not due to the yellow fever virus. Unless a note is made to the effect that the control groups receiving immune serum and virus gave unsatisfactory results, it is to be assumed that the mice in these groups were protected. From the record cards the results are then classified as "protection," "no protection," "inconclusive," or "unsatisfactory," in accordance with the requirements of the guide given in Table IV. Unless a very few deaths in the "protection" groups and a similar number of survivals in the "no protection" groups were allowed, the

517	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	May 26, 1931
Mouse Group	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Date
1137												+				
Serum																
F-142																
Virus																
Control for																
Control Groups																
529-536																
6/6 (1/11) +																
Result																
Source of Virus <i>See card 511</i>																
Inoculum: Intracerebral-Starch 27, 0.03 cc.																
Intraperitoneal - Virus 20%, 0.2 cc.																
Serum 100%, 0.4 cc.																
Mouse Strain <i>K</i>																

Key to symbols
used in records

+ = Died

‡ = Killed when
sick

‡ = Killed when
well

S = Sick

M = Moribund

F-142 = French

strain of virus,
142nd passage
in mice

6/6 (1/11) = 6 of 6
mice were pro-
tected in test;
1 of 11 survived
in normal se-
rum controls

+ = Serum pro-
tected

± = Result incon-
clusive

- = Serum did
not protect

U = Test unsat-
isfactory

Heavy vertical
lines bound
time zone within
which deaths
are considered
significant

529	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	May 26, 1931
Mouse Group	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Date
Immune																
human																
Serum																
F-142																
Virus																
Control for																
511-528																
Control Groups																
(5/5)																
Result																
Source of Virus <i>See card 511</i>																
Inoculum: Intracerebral-Starch 27, 0.03 cc.																
Intraperitoneal - Virus 20%, 0.2 cc.																
Serum 100%, 0.4 cc.																
Mouse Strain <i>K</i>																

533	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	May 26, 1931
Mouse Group	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Date
Normal																
monkey																
Serum																
F-142																
Virus																
Control for																
511-528																
Control Groups																
(0/6)																
Result																
Source of Virus <i>See card 511</i>																
Inoculum: Intracerebral-Starch 27, 0.03 cc.																
Intraperitoneal - Virus 20%, 0.2 cc.																
Serum 100%, 0.4 cc.																
Mouse Strain <i>K</i>																

FIG. 1. Part of record of test of a serum by the intraperitoneal protection test in mice, to determine protective power against yellow fever. Upper card contains record of test; other two show that of half the immune and normal serum controls.

"inconclusive" and "unsatisfactory" results would be excessively numerous and many tests would have to be repeated. Our experience leads us to consider the requirements in the table to be conservative. Very rarely there may be a number of deaths on the 11th or 12th days after inoculation, in which case it may sometimes be justified to classify the result as "inconclusive" when it would otherwise be "protection." Ordinarily the deferring of death beyond the 10th day shows a considerable protective action of the serum and would have almost the same significance as a survival. If the amount of serum will permit, the tests of the specimens in the "inconclusive" and "unsatisfactory" groups are repeated, and if definitely positive or negative results are then obtained they are accepted, but

TABLE IV
Guide for Interpretation of Results of the "Intraperitoneal Protection Test" in Mice

No. of mice living and well 4 days after inoculation	No. of deaths or survivals allowed among these mice 5 to 10 days after inoculation if the report is to be "protection" or "no protection," respectively*	
	Protection (+)	No protection or negative (-)
	No. of deaths allowed	No. of survivals allowed
1, 2, 3	Result "unsatisfactory" (U) in any case	
4		
5, 6		
7, 8, 9		
10, 11, 12		
	0	0
	1	1
	2	2
	3	3

* If there is neither "protection" nor "no protection" but the controls are satisfactory, the result is classed as "inconclusive" (\pm). If the results with the normal serum and known immune serum controls are not "no protection" and "protection," respectively, in conformity with the standards of the table, all the tests to which the controls relate are classed as "unsatisfactory" (U). If possible, all "inconclusive" and "unsatisfactory" tests are repeated.

the protection ratios for both tests are shown in the tabular reports to the senders of the sera. The "unsatisfactory" results include those in which the controls are unsatisfactory and those in which fewer than four mice are alive and well 4 days after inoculation.

Bacteriological and Pathological Examinations.—Ordinarily the identification of the probable cause of death of the mice in the routine tests rests on the known source of virus and the fact that in the controls the known immune yellow fever serum protects the mice and the normal serum does not. If there are circumstances suggesting that deaths may be due to contamination of the individual specimen of serum, cultures may be taken of the serum and histological examinations may be made of the brains of the mice. Such difficulties are rarely encount-

ered if the sera are collected with great care and in sufficient quantity. The test is hardly worth while if a shipment of sera contains many specimens that are contaminated or below 3 cc. in amount.

Demonstration of the Presence of Virus in the Blood of the Mouse

The intraperitoneal protection test in mice was devised on the supposition that yellow fever virus injected into the peritoneal cavity quickly found its way into the blood stream and was carried through the blood vessels of the brain. Theiler had shown that the blood contained little or no virus during the course of the disease, and we were unable to find virus in the blood after symptoms had appeared.

To demonstrate the virus in the circulating blood after inoculation and its absence during the course of the yellow fever encephalitis, we carried out the experiments recorded in Table V.

In the principal experiment, mice were each given intraperitoneal injections of 0.2 cc. of a 20 per cent suspension of brain virus, French strain, 124th passage in mice, together with 0.4 cc. of normal human serum. The amounts of virus and serum were the same as were then being used in our routine protection tests. The mice were divided into two series, A and B. One group of mice of Series A was bled from the heart under ether anesthesia 24 hours after the injection. Other groups were similarly bled 48, 72, 96, and 120 hours after inoculation. The blood of each group was heparinized and mixed and then injected intracerebrally in amounts of 0.03 cc. into groups of normal mice as a test for the presence of virus in the injected blood. The results are shown in Table V. A considerable but irregular proportion of the mice inoculated died within the usual time period for deaths from experimental yellow fever after intracerebral inoculation of fixed virus (4 to 9 days after inoculation).

The mice of Series B were bled in the same way, but the fresh untreated blood of each individual mouse was injected immediately into a group of normal mice. As before, the injections were intracerebral and consisted of 0.03 cc. of blood. The times of bleeding were the same except that the bleedings after 120 hours were omitted. The results were similar to those of Series A.

In the first supplementary test recorded in Table V, mice were inoculated as in the principal test. The virus was of the French strain, 108th passage in mice, and 0.2 cc. of a 20 per cent suspension was injected intraperitoneally into each mouse. The mice were bled 24 hours later and the blood was pooled and allowed to clot. The serum was injected into normal mice intracerebrally in amounts of 0.03 cc. The result was almost identical with that obtained in Series A of the principal experiment with the inoculation of pooled whole blood after the same time interval. Of eleven mice inoculated with the serum and surviving on the third day, nine succumbed.

In the second supplementary test, the mice were inoculated intraperitoneally with the same amount of virus, French strain, 109th and 110th passages in mice mixed with normal human serum, but they were given also an intracerebral

TABLE V
Recovery of Yellow Fever Virus from the Circulating Blood of Mice at Varying Intervals after Massive Intraperitoneal Inoculation

	Hours between inoculation and bleeding of source animal					
	24 (1 day)	48 (2 days)	72 (3 days)	96 (4 days)	120 (5 days)	144 (6 days)
<i>Principal Experiment, Series A</i>						
No. of mice bled as source (blood heparinized and pooled)	5	5	4	4	2	
No. of mice inoculated intracerebrally with the mixed blood and alive 3 days later	11	10	10	10	10	
No. of these mice dying or moribund 4 to 9 days after inoculation	8	3	1	6	7	
<i>Principal Experiment, Series B</i>						
No. of mice bled as source (untreated blood of each used separately)	5	5	5	4		
No. of mice inoculated in groups with these separate bloods and alive 3 days later	25	22	24	19		
No. of these mice dying or moribund 4 to 9 days after inoculation	8	9	13	8		
<i>Supplementary Experiments</i>						
No. of mice bled as source (blood pooled)	6*					
No. of mice inoculated with the mixed serum and alive 3 days later	11				4†	
No. of these mice dying or moribund 4 to 9 days after inoculation	9				16	
					0	

* First supplementary experiment.

† Second supplementary experiment.

injection of 0.03 cc. of 3 per cent starch solution to localize the virus and permit infection to take place. In this way it was hoped to find out whether the virus would disappear by the 6th day after injection, and, if it did, to ascertain whether it would reappear with the appearance of symptoms. Four of the mice which were

moribund or definitely ill 6 days after inoculation were anesthetized and bled from the heart. The mixed serum was injected intracerebrally, in the amount used in the previous experiments, into sixteen normal mice. All of these mice remained well throughout the observation period of 14 days. The four mice whose blood had been taken for injection were killed and their brains were ground up together to make a 20 per cent suspension. Twelve mice were inoculated intraperitoneally each with 0.2 cc. of this suspension mixed with 0.4 cc. of normal human serum. Twelve other mice were similarly inoculated, but a known human immune serum was substituted for the normal serum. All the mice were given also 0.03 cc. of 3 per cent starch solution intracerebrally. Of the mice receiving normal serum, one died early, and the remaining eleven died from 5 to 8 days after inoculation. Of the twelve receiving immune serum, all but one remained well for the entire observation period of 14 days. Thus was shown the presence of the virus in the brain and its identity as yellow fever virus. Evidently the virus had disappeared from the blood before the 6th day after inoculation and had not reappeared with the onset of the encephalitis and the production of much yellow fever virus in the brain tissues.

While the evidence from these several experiments strongly suggests that yellow fever virus was present in the circulating blood of mice every day from the 1st to the 5th after intraperitoneal inoculation and caused all, or most of, the deaths in the mice inoculated with the blood specimens, the presence of the virus has been confirmed by histological examinations and subinoculations only for specimens of blood taken 24 hours, 48 hours, and 96 hours after inoculation. The evidence follows.

Serum Taken after 24 Hours.—(First supplementary experiment, Table V.) One of the mice which had been inoculated with the serum was killed when moribund, and part of its brain was examined histologically. There were definite lesions of encephalitis, typical of those seen in yellow fever in the mouse. Half of the same brain was ground up with half the brain of another mouse in the same group, and normal mice were inoculated intracerebrally with the suspension. Of twelve mice alive on the 3rd day after inoculation, eleven died from 4 to 9 days after inoculation, and the brain of one was examined and found to show pronounced and typical lesions of yellow fever encephalitis. Further subinoculations were made similarly from this group, and of seventeen mice alive on the 3rd day, fifteen died from 4 to 9 days after inoculation. The brains of two other mice of the group receiving the 24 hour serum were ground up together and used in inoculating eighteen mice intracerebrally. All of them died 4 to 6 days later. The brains of two were examined and found to show lesions of encephalitis.

Blood Taken after 48 Hours.—(Principal experiment, Series B, Table V.) Brain tissue of one of the mice which had received 48 hour blood was injected intracere-

brally into normal mice. Of ten alive on the 3rd day, all died or were moribund 5 to 6 days after inoculation. The brains of two were examined histologically and found to present lesions of encephalitis consistent with those of yellow fever.

Blood Taken after 96 Hours.—(Principal experiment, Series B, Table V.) One of the mice which had been inoculated with 96 hour blood was killed when moribund, and part of its brain was examined histologically. The lesions of encephalitis were present and considered to be those of yellow fever. The remainder of this brain was ground up with the brain of another mouse in the same group, and twelve mice were inoculated intracerebrally with the suspension. All died or were moribund 5 days later. The brain of one was examined and found to present the picture of yellow fever encephalitis.

From these observations it seems that the mouse is unique, among the animals so far investigated, with regard to the circulation of yellow fever virus in its blood, inasmuch as the virus in the blood disappears before symptoms begin and does not reappear with the onset of obvious disease. After massive intraperitoneal inoculation the virus appears promptly in the blood and remains for several days. If the virus in the circulating blood is given an opportunity to attack the brain through a local injury and produces disease on the 6th day after inoculation, the virus is absent from the blood at the time the symptoms begin, although it is abundant in the brain tissue. It would seem that the mouse, like many other animals, permits the injected virus to circulate for a time regardless of whether a manifest infection is to take place, and that the virus disappears from the blood after a few days. If the encephalitis of yellow fever occurs later, the virus does not reappear in the blood. We do not yet know whether protective substances against yellow fever are produced in the mouse by intraperitoneal inoculation in the absence of the production of yellow fever encephalitis, but protective substances have been shown to appear in several other resistant warm blooded animals after such inoculation (13). There is a possibility that the rise of antibodies in the blood frees the blood of virus while permitting virus to continue its development or to persist in the central nervous system, just as yellow fever virus might remain for a time in the liver of an infected monkey after it had disappeared from the circulation with the rise of antibodies. We do not know whether the virus in the circulating blood increases in amount or is simply the virus introduced by massive inoculation. Whatever the process is that permits virus to be recov-

ered from the blood, it seems evident that it does not depend on the production of the encephalitis which characterizes experimental yellow fever in the mouse.

Differences in the Susceptibility of Strains of Mice to Yellow Fever Virus

In the first months of our study, while using the strain of white mouse bred at The Rockefeller Institute for general purposes, we were disappointed in the seeming low virulence of the virus strains used. Even the fixed French strain, which had become so highly virulent in the hands of Theiler (11), allowed many of the mice to survive after intracerebral inoculation. We found the mortality too low at that time to permit the development of a satisfactory protection test in mice. In October, 1930, there was an abrupt rise in the mortality to approximately 100 per cent, and we observed that this coincided with the purchase of mice from Dealer K at a time of shortage of the Institute stock. We later secured strains from other sources and tested their susceptibility. Through the kindness of Dr. Carrel we received mice of the Dilute Brown strain and of the albino Swiss strain. The latter strain had been brought to the Institute by Dr. Clara Lynch in 1926 from the laboratory of Dr. de Coulon of Lausanne, Switzerland. Mice were secured also from three dealers, K, M, and D.

The results of comparative tests for susceptibility are shown in Table VI, and may be briefly summarized. Of the three pure strains, the Swiss and the Dilute Brown were found to be highly susceptible and suitable for use in the protection test. The mortality was nearly 100 per cent for both strains, but the incubation period was slightly shorter in the Dilute Brown mice than in the Swiss.

The Institute stock strain was remarkably resistant. This resistance was manifest even in suckling mice. Unlike adult mice, very young mice will ordinarily contract yellow fever encephalitis when inoculated by intraperitoneal injection in the absence of cerebral injury, as has been shown by Theiler (11). Suckling mice of the Institute stock, however, were found to be highly resistant to intraperitoneal inoculation, while similar mice from Dealer K were very susceptible.

This experience brings out the importance of using a pure strain of mouse of known susceptibility in yellow fever studies and protection

TABLE VI

Comparative Tests of Strains of Mice for Susceptibility to Yellow Fever Virus of the French Strain after 109 to 138 Passages in Mice

Comparative tests		Pure strains			Mixed strains		
		Swiss	Dilute Brown	Institute stock	Dealer K	Dealer M	Dealer D
<i>Intracerebral Inoculation of Young Adult Mice*</i>							
Test 1	No. of mice.....			55		52	
	Percentage dead or moribund 4 to 9 days after inoculation....			65%		96%	
Test 2	No. of mice.....			48	95		
	Percentage dead or moribund....			50%	98%		
Test 3	No. of mice.....				53		54
	Percentage dead or moribund....				100%		81%
Test 4	No. of mice.....	30	30		30		
	Percentage dead or moribund....	100%	100%		83%		
Test 5	No. of mice.....	23	24		23	22	
	Percentage dead or moribund....	100%	100%		100%	100%	
	No. dead or moribund on 4th day after inoculation.....	3	17		7	2	
	No. dead or moribund on 5th day after inoculation.....	19	7		16	20	
<i>Intraperitoneal Inoculation of Suckling Mice 13 to 16 Days Old†</i>							
Test 6	No. of mice.....			13	18		
	Percentage dead or moribund 5 to 10 days after inoculation.....			0%	83%		
	No. dying 11 to 15 days after inoculation.....			4	1		

* Inoculum: 10 per cent suspension of brain of moribund mouse in isotonic sodium chloride solution. Amount injected intracerebrally: 0.03 cc.

† Same inoculum as used intracerebrally. Amount injected intraperitoneally into suckling mice: 0.1 cc.

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tests. Unfortunately, this is not always possible, and we are still compelled to buy reasonably susceptible mice of unknown and mixed heredity from dealers, while an insufficient but increasing supply of mice of the Swiss strain is being bred for us.

Titration of Sera for Protective Power

The technic of the intraperitoneal protection test in mice is well adapted to the titration of sera for their protective power against yellow fever. It is merely necessary to substitute serial dilutions of the serum for the full strength serum in the test. The methods of recording and interpreting remain the same.

We may take as an example the results of a titration in a study of the persistence of yellow fever immunity (9). The serum was from a woman who had had yellow fever 78 years before. The upper card in Fig. 1 shows the record of the full strength serum in this titration. The controls were satisfactory; the normal serum controls permitted only one of eleven mice to survive. The protection ratios and results for the several dilutions were: dilution 1:1, 6/6 (+) meaning that six mice out of six survived; 1:2, 6/6 (+); 1:4, 6/6 (+); 1:8, 4/5 (+); 1:16, 4/6 (\pm); 1:32, 4/6 (\pm); 1:64, 1/6 (-); 1:128, 1/6 (-). By the rigid standard of Table IV, the specimen of serum protected in dilutions up to 1:8, and gave inconclusive results at 1:16 and 1:32, and gave no protection in higher dilutions. The titer would be 1:8.

Sensitivity of the Test

The intraperitoneal protection test in mice is much more sensitive than the protection test as made in monkeys. Sera which protect monkeys also protect mice, but many which protect mice do not protect monkeys.

The serum which was mentioned above as protecting mice in a dilution of 1:8 was tested also in two monkeys. One was given 3 cc. of the serum per kilo of body weight and the other 1.5 cc. per kilo by intraperitoneal injection, and each received a subcutaneous injection of 0.4 cc. of Asiatic yellow fever virus in monkey blood 6 hours later. All of three control animals died of yellow fever. The test animals both developed fever and recovered, showing that they had received definite protection but of a low degree. The sera of several persons who have recovered in recent years from laboratory infections usually protect monkeys completely when as much as 1 cc. per kilo is injected and protect mice when the dilution of the serum is 1:50 or 1:100. The intraperitoneal test in mice has been found sufficiently sensitive to reveal active immunity in persons and monkeys successfully protected

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STUDIES ON THE FILTRABILITY OF BACTERIUM GRANULOSIS

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The question of whether a filtrable virus plays any rôle in the etiology of trachoma has long been a subject of investigation.

As early as 1905, Pfeiffer and Kuhnt (1) unsuccessfully attempted to produce trachoma in man with filtrates of trachomatous materials. In 1907, Fermi and Repetto (2) inoculated twenty-three humans and two apes with filtrates derived from the conjunctival material of fifty fresh trachoma cases. They used Berkefeld filters which had been tested for permeability with *B. prodigiosus*, and limited the time of filtration to 5 minutes. None of those inoculated with filtrates became infected, but the controls, injected with the unfiltered material, showed conjunctival follicles which, after 2 months, had resisted cauterization treatment. In 1910, Hess and Römer (3) excised twenty culs-de-sac of trachomatous individuals and prepared a filtrate with which they inoculated three *cynocephalus* monkeys. With the unfiltered material, they injected three other monkeys. The animals receiving the filtrate showed no reaction after several months' observation; but those which had been given the unfiltered suspension developed granulation of their conjunctivae in 4 days. Hess and Römer concluded that trachoma was not caused by a filtrable virus. As quoted by Weiss (4), Bajardi, as well as Lindner, reported negative results from inoculation of trachomatous filtrates in monkeys.

Nicolle (5) and his coworkers, in 1913, filtered trachomatous tissue suspensions through a Berkefeld filter of the "most permeable type" (presumably Type V), especially reconstructed by them for small amounts of material. The candle was impervious to *Vibrio cholerae*. The filtrate was injected into the conjunctiva of a magot. As a control, another magot was similarly inoculated with the unfiltered material. After 43 days, the first animal showed what was considered to be "typical" trachoma. This condition disappeared after a few months. The control animal exhibited granulations on the tarsal conjunctiva in 20 days. A *Macacus rhesus* monkey, also serving as a control, failed to react to the inoculations. In a second experiment, Nicolle and his associates followed the methods of the first test, save that material from two trachomatous individuals was pooled and a chimpanzee was injected with the filtrate and a magot with the unfiltered material. The chimpanzee showed granulations of the conjunctiva in 14 days; the control magot in 10 days. 50 days after the inoculation of the chimpanzee, the maximum reaction was reached, but on the 80th day the lesions had disappeared.

Trapesontzewa (6), in 1930, prepared a filtrate of the pooled conjunctival material from sixteen trachomatous patients and inoculated three blind persons. The filtration was made through a filter which permitted small amounts of fluid to be used. During 6 months' observation none of the inoculated individuals developed lesions other than an initial, slight, transitory edema. In a second experiment, the pooled and filtered conjunctival material from twelve persons with trachoma was inoculated subconjunctivally into three other individuals, including the experimenter. The only effect was a slight transitory edema, lasting not more than 36 hours. Trapesontzewa repeated this experiment twice, using material from twenty-four trachoma patients, and obtained a similar effect in both instances. No mention was made of the infectivity of the unfiltered material.

Recent work has brought out the fact that several species of bacteria are capable of passing through filters of sorts which had been supposed to exclude them. Grinnell (7) demonstrated that one has but to alter the physical conditions of a suspension of an organism in order to affect its filtrability. Cuénod (8), one of Nicolle's coworkers in the filtration experiments of the etiological agent of trachoma, says: "That trachoma can be induced experimentally by a filtrate is not opposed to the possibility of a bacterium visible microscopically."

The positive evidence that trachoma is caused by a filtrable virus rests upon the two experiments of Nicolle and his associates, in which a filtrate was active in one animal in each test. Furthermore, the "virus" of these investigators is described as being rapidly destroyed by heat at 56°C., by desiccation, and by glycerol (in 8 days). Such sensitiveness is not usual with ultramicroscopic viruses.

The following questions are unsettled (*a*) whether the incitant of trachoma is filtrable; (*b*) whether *Bacterium granulosis*, which Noguchi (9) and others have shown to be associated with trachoma, is filtrable, and, if so, whether it is pathogenic in this state, and (*c*) whether, as Cuénod and Nataf (10) suggest, a filtrable virus may be adsorbed to *Bacterium granulosis*. Because of the present uncertainty with regard to these matters, investigators believing that trachoma is due to a filtrable virus despite the greater amount of evidence to the contrary, we have made filtration tests with trachomatous materials and with *Bacterium granulosis*.

Materials and Methods

Preparation of Human and Monkey Material.—There were available for study the tarssectomized tissues from eight patients with trachoma of from 2 to 11 years' duration.¹

¹ For the clinical material we are very grateful to Dr. Martin Cohen of New York City.

A suspension was made of the tissue from each case by grinding for a few minutes in a sterile mortar with 2 to 5 cc. of sterile physiological saline solution. (In Cases V and VI the suspensions were made in Dunham's peptone water medium instead of in saline.) Prior to filtration, 0.2 cc. of this material was inoculated subconjunctivally into one upper cul-de-sac of each control *Macacus rhesus* monkey, and a small amount of the suspension was set aside for cultivation of *Bacterium granulosis* after the manner of Noguchi (9). Of the filtrate, prepared as described below, 0.2 cc. was inoculated subconjunctivally into each test animal and all of the remainder was used for cultures which were made by adding from 2 to 5 drops of the filtrate to tubes of leptospira medium. The tubes were kept for at least 3 weeks before they were discarded as negative.

The conjunctivae of two *Macacus rhesus* monkeys with the disease induced by inoculation of human tissues or *granulosis* cultures were used for similar filtration experiments. The conjunctivae from the upper and lower lids of one eye of each were removed,² and prepared for inoculation and filtration in the same manner as were the human tissues.

Standardization of Filters.—Berkefeld V filters were used. The permeability of the filters was tested with cultures of *B. prodigiosus* both before and after the filtration proper. This was done as follows:

A suspension of *B. prodigiosus* of ground glass opacity was prepared by washing growths of the organism from agar slants with sterile physiological saline solution. About 5 cc. of this suspension were introduced into the filter, under a pressure difference of 25 to 35 cm. Hg. In no test did the filtration time exceed 3 minutes. The filtrate thus obtained was inoculated into tubes of plain broth, which were incubated at 37°C. and not discarded as negative until after at least 3 days' incubation. After cleaning and resterilization, these filters were used for the experiment. Immediately afterwards they were changed to other flasks and the suspension of *B. prodigiosus* was again introduced. If, in either test, *B. prodigiosus* passed through the candle, the results of the experiment were thrown out. Almost one-half of the new Berkefeld V filters examined allowed the passage of the organisms.

Method for the Filtration of Bacterium granulosis.—Cultures of *Bacterium granulosis* from 4 to 7 days old, grown on slants of horse blood agar containing a mixture of carbohydrates (11), were employed. To each tube, 8 cc. of sterile physiological saline solution were added, the culture was scraped from the surface of the agar, and the tube shaken vigorously until a suspension of ground glass opacity was obtained. 5 cc. of this suspension were removed and put into a Berkefeld V

² Full ether anesthesia was used for all operative procedures.

filter, standardized as described above. The filtration was aided by a pressure difference of 35 to 40 cm. Hg and required no more than 3 minutes, usually about 45 seconds. The filtrate was cultured in tubes of leptospira medium, not more than 1 cc. being added to each tube. These cultures were incubated at 28°C. and examined daily. Before being discarded as negative, they were observed for at least 3 weeks.

Tests for a Filtrable Agent of Trachoma

Human Trachoma.—With the material from eight cases of human trachoma, seven tests were made, the suspensions from the first two cases being pooled.

Cases I and II.—Suspensions from two cases of human trachoma were pooled and filtered. The filtrate was inoculated into three *Macacus rhesus* monkeys. One of these showed a transitory, slight follicular reaction; another had a mild follicular conjunctivitis, which had disappeared in 12 weeks, and a third showed no effect. Two control animals, inoculated with unfiltered material, were used. One of these was not affected. The other showed a mild, transitory type of reaction, with a few follicles in both eyes. *Bacterium granulosis* was recovered from the unfiltered suspension. The cultures of the filtrate were negative.

Case III.—Three monkeys were inoculated with the filtrate. None of them was affected. Tarsectomy was performed on one animal and the material was cultured for *Bacterium granulosis*. The organism was not recovered.

Two control animals, inoculated with unfiltered tissue suspension, were used. One of them showed after 8 weeks a slight follicular reaction which had cleared up in 12 weeks. The other was unaffected. *Bacterium granulosis* was not recovered from the original suspension, nor was any organism recovered from the filtrate.

Case IV.—Three monkeys were inoculated with the filtrate. All remained negative. The material derived from one of them was cultured for *Bacterium granulosis* but the organism was not recovered. Of two control animals, one developed the characteristic experimental disease; the other showed a transitory, mild follicular reaction. *Bacterium granulosis* was recovered from the human case and also from the more severely infected control monkey. The culture of the filtrate was negative.

Case V.—The filtrate was tested in three monkeys. Two of them remained negative. The other animal exhibited in 2 weeks characteristic trachomatous conjunctivitis, which in 8 weeks had largely subsided. *Bacterium granulosis* was not recovered from this animal. Of two control animals, one showed no reaction; the other showed in 8 weeks a few follicles in the inoculated eye, which have persisted. *Bacterium granulosis* was not recovered from the human case. The filtrate culture was negative.

Case VI.—Two animals received the filtrate. Both remained negative. Two

control animals developed experimental trachoma. *Bacterium granulosis* was recovered from the human case and from the one control monkey which was examined. The filtrate culture remained negative.

Case VII.—Two monkeys were inoculated with the filtrate. Neither of them was affected. Of two controls, one remained negative; the other developed a persistent, mild follicular reaction. *Bacterium granulosis* was not recovered from this animal. Culture of the human tissue yielded *Bacterium granulosis*. The filtrate culture was negative.

A decision as to the positive or negative character of the results was rendered difficult by the mild follicular type of reaction which some of the animals exhibited. This condition either cleared within 2 to 6 weeks, or persisted for over 12 weeks. The exact significance of this transitory type of follicular conjunctivitis is not yet clear; it may possibly have some relation to the experimental disease. Inasmuch as true experimental trachoma is progressive and persistent, only those reactions which endured for long periods of time could, on the basis of our present knowledge, be considered positive (Table I).

Monkey Trachoma.—Two *Macacus rhesus* monkeys (AA and BB) which showed the characteristic experimental disease as result of inoculation with *Bacterium granulosis* were used for this group of tests. Conjunctival tissue was removed from the animals and prepared and filtered in the same manner as with human tissue.

The filtrate from *Macacus rhesus* AA was injected into two monkeys. Neither developed granular conjunctivitis. One control animal inoculated with unfiltered tissue suspension developed experimental trachoma, and the other control showed a persistent, although mild, follicular conjunctivitis. Culture of the conjunctiva of the latter animal revealed no *Bacterium granulosis*, but that from *Rhesus* AA yielded the organism. The culture of the filtrate remained sterile.

The conjunctival suspension from *Rhesus* BB was inoculated into two monkeys. Of these, one developed characteristic trachomatous conjunctivitis, and from its conjunctiva *Bacterium granulosis* was isolated. The filtrate was inoculated into three monkeys, none of which showed conjunctival lesions. The filtrate failed to yield cultures of *Bacterium granulosis* (Table II).

A summary of the results of experiments on filtration of tissue suspensions of human and experimental trachoma reveals that only one of twenty monkeys inoculated with filtrates yielded a positive reaction, while nine of sixteen animals inoculated with unfiltered material showed characteristic trachomatous conjunctivitis. That all

TABLE I
Filtration Experiments with Human Tissue

Case	<i>B. granulosis</i> culture from human case	Unfiltered suspension			Filtered suspension				<i>B. granulosis</i> culture from monkey's conjunctiva
		Monkey a	Monkey b	<i>B. granulosis</i> culture from monkey's conjunctiva*	Culture of filtrate	Monkey c	Monkey d	Monkey e	
I and II	Positive (A. P.)	Negative	Negative		Negative	Negative	Negative	Negative	Negative Monkey c
III	Negative	Positive	Negative		Negative	Negative	Negative	Negative	Negative Monkey c
IV	Positive	Negative	Positive	Positive Monkey b	Negative	Negative	Negative	Negative	Negative Monkey d
V	Negative	Positive	Negative	Positive Monkey b	Negative	Negative	Positive	Negative	Negative Monkey d
VI	Positive	Positive	Positive	Positive Monkey b	Negative	Negative	Negative	Negative	Negative Monkey d
VII	Positive	Negative	Positive	Negative Monkey b	Negative	Negative	Negative	Negative	Negative Monkey c

* Blank spaces indicate that no test was made.

TABLE II
Filtration Experiments with Monkey Tissue

Case	<i>B. granulosis</i> culture from case	Unfiltered suspension			Filtered suspension				<i>B. granulosis</i> culture from inoculated monkey conjunctiva
		Monkey a	Monkey b	<i>B. granulosis</i> culture from inoculated monkey conjunctiva	Culture of filtrate	Monkey c	Monkey d	Monkey e	
AA	Positive	Positive	Positive	Negative Monkey a	Negative	Negative	Negative	Negative	Negative Monkey c
BB	Positive	Positive	Negative	Positive Monkey a	Negative	Negative	Negative	Negative	Negative Monkey c

Macacus rhesus monkeys do not develop trachoma after subconjunctival injections of trachomatous tissue suspensions has already been pointed out by us (12) and by others. The significance of the single positive result with a filtrate remains to be determined.

Filtrability of Bacterium granulosis

In an attempt to determine whether or not *Bacterium granulosis* was capable of passing through tested Berkefeld V filters, thirty-one filtration tests were performed on ten strains of the organism. One

TABLE III
Filtration of Bacterium granulosis

Strain	Berkefeld filter	Suspension fluid		Strain	Berkefeld filter	Suspension fluid		Strain	Berkefeld filter	Suspension fluid		Strain	Berkefeld filter	Suspension fluid	
		Normal saline	Hormone broth			Normal saline	Hormone broth			Normal saline	Hormone broth			Normal saline	Hormone broth
Alb. No. 1	V	-	+	EL	V	-	-	L-9	V	-	-	L-6	V	+	-
Alb. No. 1	V	+	+	HD	V	-	-	L-5	V	-	-	L-6	V	+	-
MC	V	-	+	HD	V	-	-	L-5	V	+	-	L-6	V	+	-
MC	V	-	+	HD	V	-	-	Ariz. No. 3	V	-	-	L-6	V	+	-
AP	V	-	-	HD	V	-	-	L-6	V	+	+	L-6	N	-	-
AP	V	-	-	510	V	+	+	L-6	V	+	+				
EL	V	-	-	510	V	+	+	L-6	V	+	+				

culture was derived from one of the cases (I) mentioned above. Another was obtained from a monkey which had been inoculated with tissue from a human case not included in this series, which also yielded *Bacterium granulosis*. The remainder were old strains that had been in our possession for from 1 to 3 years.

Two tests each were made on three of the strains (including that from Case I) and no growth occurred in the filtrate. In the case of the two other strains, each passed through the filters once in two trials. Two more strains failed to come through in one test for each. The culture derived from the monkey was filtrable both times in two tests. One strain (L-6) passed through the filters in each of six trials. The experiments were repeated, using the last strain mentioned,

together with one that had filtered once out of two tests, and one that had twice failed to pass the filters. Hormone broth was used for suspension instead of normal saline solution, since it has been found by Ward and Tang (13) to favor the filtrability of herpes and vaccine viruses; and by Grinnell (7) to facilitate the filtration of *Bacillus prodigiosus*. Two tests were made on each strain, and in all six trials the organisms were recovered from all tubes which had been inoculated with the filtrate. The strain which consistently passed the Berkefeld V filters was tested through an N filter but was not then found to be filtrable (Table III). Less than half of the inoculated leptospira tubes of the "filtrable" strains showed a growth, in some instances only one tube of ten. Hence one may infer that very few organisms were present in the filtrate.

We have found, therefore, that four out of ten strains of *Bacterium granulosis* were filtrable through Berkefeld V candles. Only two of these strains filtered consistently, and it is evident that even in these cultures the organisms were present in the filtrate in small numbers.

Action of Filtrates of Bacterium granulosis

It has not been found possible up to the present time to pass a pathogenic strain of *Bacterium granulosis* through tested filters.

Filtrates of pathogenic strains of the organism, bacteriologically sterile, were inoculated into two monkeys, neither of which developed conjunctivitis. In these tests five control animals, inoculated with unfiltered suspensions of growths, developed the characteristic experimental disease. In another similar experiment, two monkeys injected with filtrates of *granulosis* cultures were likewise unaffected.

DISCUSSION AND SUMMARY

The evidence hitherto reported concerning the filtration of trachomatous material, and inoculation of man and monkeys with the filtrates points to the conclusion that the incitant of trachoma is not, as a rule, filtrable. Our findings confirm this view and indicate further that no virus causing the disease is adsorbed to *Bacterium granulosis*. On the other hand, *Bacterium granulosis* itself in heavy suspensions is irregularly filtrable through Berkefeld V candles, like some other bacteria (14), but it is present in the filtrates in only small numbers. When suspensions were used of trachomatous human and monkey tissues, which contain much fewer organisms than do actual cultures, *Bacterium granulosis* was never recovered from the filtrates.

The conception that trachoma is a disease caused by an ultra-microscopic virus is based on (a) the positive results of filtration in two animals, as reported by Nicolle and his coworkers, and (b) the presence of so called "inclusion bodies" in some of the cells of the lesions. One can state definitely that the evidence is now greatly against the filtrability of the etiological agent of trachoma. Furthermore, filtrability does not in itself suffice for the classification of an agent as an ultra-microscopic virus. Concerning (b), a vast literature has accumulated which indicates that the "inclusion bodies" of trachoma are not specific for the disease and that the bodies themselves may be bacterial in origin (15). We have not as yet found bodies of the kind characteristic of many filtrable viruses in the tissues of man or of monkeys with the experimental disease.

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THE TRANSMISSION OF THE VIRUS OF MEXICAN TYPHUS FROM RAT TO RAT BY POLYPLAX SPINULOSUS

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In a recent communication, the writers (1) have reported upon the discovery of the virus of Mexican typhus fever in wild rats trapped in the City of Mexico. Two strains of the virus were obtained by injecting into guinea pigs, intraperitoneally, emulsions of brain material of rats trapped upon premises in which typhus fever had appeared during the epidemic which was going on at the time the work was done, that is, April, 1931. Particulars concerning this investigation are given in the paper referred to.

Our own observation, as cited, taken together with the recent observations of Dyer and his collaborators (2), definitely establish the wild rat as a reservoir of the disease and probably the most important means by which the infection is kept alive in communities during interepidemic periods. The problems which now arise are, in the first place, the exact method by which the disease is, under practical conditions, transmitted from rat to man, and, in the second place, the method of transmission from rat to rat. There is, of course, no question in our minds concerning the importance of the human louse in transmission from man to man in times of epidemic, a fact first established by Nicolle.

In order to throw additional light upon the two problems stated above we began, together with our search for the virus in the rats themselves, to carry out surveys of the ectoparasites found upon the captured rats, classifying the insects, and injecting them into guinea pigs, in the hope of discovering whether any of them, and if so which ones, were of importance in connection with virus transmission.

The present paper deals particularly with the question of transmission of typhus virus from rat to rat, that is, the mechanism by which the endozoic rat disease is kept going.

Insect Distribution

In an insect survey of about 100 wild rats, the following arthropods were found:

Fleas: <i>Xenopsylla cheopis</i>	91
<i>Ceratophyllus fasciatus</i>	11
<i>Leptopsylla musculi</i>	13
<i>Ctenocephalus felis</i>	1
Mites: <i>Laelaps echidninus</i> , numerous on several rats.	
Lice: <i>Polyplax spinulosus</i> , numerous to very numerous on many rats.	

The distribution of fleas on the wild rats was very irregular, even when the rats were caught at the same time and on the same premises. Some of them harbored numerous fleas, others one or two only, and some frequently carried none at all. The same observation was made with the mites, in which a similar irregularity was observed. In regard to the rat louse (*Polyplax spinulosus*), there was a difference in distribution to some extent related to the species of rat. The rats were *Mus rattus* and *Mus decumanus*. *Polyplax spinulosus* was found on most specimens of *Mus rattus*, while its distribution on *Mus decumanus* was extraordinarily irregular. Heavily infected specimens were caught, together with specimens on which few or no lice could be found. Summarizing our insect observations, however, we may say that *Polyplax spinulosus* was by far the most frequent ectoparasite found on the rats.

Results of Inoculation into Guinea Pigs of Ectoparasites Directly Caught on Wild Rats.—Our method consisted in examining the ectoparasites of the wild rats captured and inoculating emulsions of the several species found, intraperitoneally, into male guinea pigs. Six guinea pigs were inoculated with fleas, each animal receiving the material from seven to thirty fleas. Four of these guinea pigs received fleas obtained from rats caught at the same time and in the same place with the rats from which we obtained the typhus virus. Nevertheless all these inoculations were negative. One of these guinea pigs received seven fleas from the very catch of rats from which

one of our two virus strains was isolated. All these experiments, however, were negative.

Four guinea pigs were inoculated with mites (*Laclaps echidninus*), each animal receiving the material from ten to twenty insects. These experiments also were entirely negative.

Six guinea pigs were inoculated with rat lice (*Polyplax spinulosus*), four of them receiving lice from rats caught at the Belem Prison, the locality in which the infected rats were discovered. One of the guinea pigs inoculated with these lice showed a febrile reaction suggestive of typhus, but no scrotal swelling was observed. It was killed on the 3rd day of fever but nothing characteristic was found, except a slightly edematous tunica vaginalis and several petechial hemorrhages in the polar fat of the testicles. However, guinea pigs inoculated with tunica spleen and brain of this animal remained well. Direct examination of the rat louse, therefore, was also negative.

Experiments with Polyplax spinulosus

In view of the fact that some of our wild rats of the breeding stock were heavily infested with *Polyplax spinulosus*, it seemed advisable to investigate the possible rôle of this insect as a vector of the virus of typhus from rat to rat. Many of the lice were examined for *Rickettsiae* before the experiment was started and no organisms were found which could have caused confusion.

Experiment 1.—Two rats (Nos. 1 and 2) were benzolized by the method described by Zinsser and Castaneda (3) and inoculated with tunica washings rich in *Rickettsia prowazeki*, using our original strain of Mexican typhus. 3 days after inoculation both rats had fever and looked sick. The fever lasted for 4 days in one rat (No. 1), the other (No. 2) died on the 3rd day of fever with numerous *Rickettsiae* distributed over the whole peritoneal surface. On the 12th day after inoculation it was noticed that numerous lice were dead between the hairs of the surviving rat. Six living lice were therefore removed and smears of them examined for microorganisms. An astonishing number was found in the Giemsa-stained smears of all six lice. The picture looked entirely like those observed in smears of *Pediculus humanus* infected with the virus of typhus. The intestinal contents were crowded with minute rose-red staining microorganisms, exhibiting the polar staining and morphology of *Rickettsia prowazeki*.

About thirty lice were emulsified after sterilization of the surfaces with 96 per cent alcohol and inoculated into two male guinea pigs. The reaction of these animals is shown in Table I.

TRANSMISSION OF MEXICAN TYPHUS VIRUS

Two transfers were made from Guinea Pig A, and the typical picture of Mexican typhus resulted in all animals inoculated.

Experiment 1 indicates that *Polyplax spinulosus*, feeding on rats experimentally inoculated with Mexican typhus fever, become infected, and show *Rickettsiae* in considerable numbers, if examined about 12 days after the rat has been inoculated. Such lice injected into guinea pigs give rise to the typical picture of Mexican typhus fever.

Experiment 2.—Experiment 1 was repeated with a rat not previously benzolized. *Rickettsiae* appeared in some of the lice on the 7th day after fever was noted

TABLE I

Date	Guinea Pig A	Guinea Pig B
May 9	Inoculated	Inoculated
May 10	39.4°C.	39.2°C.
May 11	39°C.	39.1°C.
May 12	39°C.	39°C.
May 13	40°C. (Scrotum swollen)	39°C.
May 14	40°C. " "	39°C.
May 15		39.9°C.
May 16	Killed for transfers	40.2°C. (Scrotum swollen)
May 17	Autopsy typical with very numerous <i>Rickettsiae</i> in tunica	39.7°C. " "
May 18		40.1°C.
May 19		39°C.
May 20		39°C.
		38.8°C.

in the rat. Of eight lice examined, large numbers were found in two of them. This inoculated rat (No. 3) was kept together with a normal rat (No. 4) in a covered glass jar. When *Rickettsiae* were found in the lice of the inoculated rat, this animal was killed. The lice began to climb to the tips of the hairs and many were collected with a camel's hair brush and put on the non-inoculated rat (No. 4). In addition to this the hair of the dead rat was clipped and left in the glass jar with the normal rat (No. 4). No fever was noticed in Rat 4 during the entire course of observation. However, the Weil-Felix reaction was performed three times with the blood of this animal, and the following results obtained:

10 days after removing Rat 3: Negative.
 12 " " " " " " Positive 1:160++.
 14 " " " " " " Positive 1:160+.

On this day it was noticed that the number of lice on this rat had decreased considerably. Of six lice examined at random, *Rickettsiae* were found in three.

The rat was now killed and an emulsion of its entire brain inoculated into a male guinea pig. Another guinea pig received twelve lice. Table II gives the reaction of these two animals (A and B).

This experiment confirms Experiment 1 in showing that rat lice, feeding on typhus-infected rats, become infected, and in addition it demonstrates that such lice may transmit the infection to another rat, by a mechanism approximating the natural relationship in which lice wander from one rat to another. Although the rat infected by means of the lice taken from the artificially inoculated animal showed

TABLE II

Date	Guinea Pig A	Guinea Pig B
June 5	Inoculated with twelve lice	Inoculated with brain of Rat 4
June 6	39.1°C.	39.8°C.
June 7	39.4°C.	39.2°C.
June 8	39.2°C.	39°C.
June 9	39.5°C.	39.2°C.
June 10	40.7°C. (Scrotum very swollen)	39.2°C.
June 11		39.6°C.
June 12	Killed. Autopsy typical with	39.7°C.
June 13	very numerous <i>Rickettsiae</i> in	39.8°C.
June 14	tunica. Cultures negative	40.2°C. (Scrotum very swollen)
June 15		40.4°C. " " "
June 16		40.4°C. " " "
June 17		40.5°C. (Swelling less.) Killed. Autopsy typical. Few <i>Rickettsiae</i> in tunica

no manifest symptoms, it developed a Weil-Felix reaction and lice fed upon this animal developed *Rickettsiae*. In spite of the negative clinical course of the rat infected by having lice feed upon him, the brain of this animal carried the virus, as was demonstrated by guinea pig inoculation.

Experiments with Mites (Laelaps echidninus)

The jar in which the rats of Experiments 1 and 2 were kept had previously been used for breeding *Laelaps echidninus* on normal white rats. It contained a large number of this species when the typhus-inoculated rats were put into the jar. On several occasions after

feeding them on the infected rats some of the mites were examined for *Rickettsiae* with negative results. When twenty of them were emulsified and inoculated into two guinea pigs they had had the opportunity of feeding on three infected rats in succession for 26 days. No reaction followed in the guinea pigs after this inoculation.

Experiment with Ornithodoros turicata

The following experiments are described in order to emphasize the specific susceptibility of *Polyplax spinulosus* as compared with other arthropods. When Rats 1 and 2 showed a febrile reaction, several specimens of *Ornithodoros turicata* were allowed to feed upon them. Eight of them engorged completely and were then kept at 32–34°C. Two were inoculated into a guinea pig (T1) 24 hours later, two into another guinea pig (T2) 48 hours later, and four into a guinea pig (T3) 9 days later. Guinea Pig T1 showed fever and scrotal swelling after an incubation period of 10 days, Guinea Pig T2 the same symptoms after an incubation period of 14 days, whereas Guinea Pig T3 remained entirely normal and was not immune to a subsequent inoculation with our passage virus. In none of the ticks used for inoculation could any organisms simulating *Rickettsia prowazeki* be found.

DISCUSSION

From the experiments described above it can be inferred that *Polyplax spinulosus* is the ectoparasite most likely to be responsible for maintaining an endozoic of typhus among rats in Mexico City. It was found on our rats more frequently and in larger numbers than any other parasite. It was the only one collected on wild rats which caused a febrile reaction in a guinea pig suggestive of typhus. Moreover, it proved to be as highly susceptible to the virus of typhus as *Pediculus humanus*. Considering its extremely small size as compared with other ectoparasites, ingesting at one feeding certainly less than one-tenth of the amount of blood taken up by *Pediculus humanus*, the regularity with which it became infected in our experiments is remarkable. There is the same enormous multiplication of *Rickettsiae* in the gut of *Polyplax spinulosus* as is seen in *Pediculus humanus* after the ingestion of an infected meal. Although we have examined smears

only, we have the impression that the *Rickettsia prowazeki* multiplies in this insect within the epithelial cells of the gut, destroying its entire lining and leading eventually to the death of the vector. Considering the very small amount of blood ingested by the rat louse, a purely mechanical transmission of the virus in our experiments can be excluded. Besides, the appearance of large numbers of *Rickettsia prowazeki* in the gut is clear evidence of the multiplication of the virus, since the etiological significance of *Rickettsia prowazeki* has been demonstrated beyond any doubt.

Our results with ticks and mites are also significant in this connection. In none of the arthropods could *Rickettsia prowazeki* be demonstrated. The two experiments with *Ornithodoros turicata* are easily explained by the survival of the virus of typhus for 2 days in the relatively large amount of blood ingested by the tick. Such survival of the virus can also be met with in specimens of blood and tissues kept in ordinary culture media.

The incubation period of Mexican typhus in guinea pigs is closely related to the amount of virus inoculated; the more virus injected the shorter the incubation period. Comparing the two guinea pigs, A and B, inoculated with lice and brain respectively in louse Experiment 2, we are justified in concluding, therefore, that the twelve lice carried more virus than the entire brain of the rat on which they had fed.

The number of lice which became infected was apparently dependent on the severity of the typhus infection sustained by the rats. While *Rickettsiae* were found in all the lice collected on the benzolized Rat 1, *Rickettsiae* appeared only in a certain percentage of the lice taken from the two other rats. The same phenomenon can be observed in *Pediculus humanus* fed on typhus cases. Although practically 100 per cent of lice, feeding on a severe case with extensive rash, will finally show *Rickettsia prowazeki*, lice put on mild cases with scant or no rash become infected much less regularly and even negative results are not infrequent. These considerations detract to some extent from the weight which certain European observers have given to the inapparent or abortive human cases of typhus, with regard to the maintenance of the infection during interepidemic periods.

The discovery of the virus of typhus in wild rats in certain com-

munities explains the occurrence of sporadic cases. The conditions for an epidemic, however, are present only when a sporadic case appears in a population sufficiently infested with lice. Whether the mechanism explains the epidemiology of Old World typhus cannot of course, be maintained without a direct study of this disease by the methods described in our preceding paper and in the present communication. For Mexican typhus fever, however, we can assert the fact that the rat is the most susceptible animal for the multiplication of the *Rickettsia prowazeki*. We have observed occasional rats not prepared by benzol or otherwise, which showed considerable numbers of *Rickettsiae* in the peritoneum within 7 or 8 days after inoculation and we have recently observed a rat in which 14 days after inoculation the entire peritoneal surface showed numerous *Rickettsiae*. The fact that the benzol so increases the susceptibility of these animals that they can be used to form the source of vaccine production has been described by us (4), the details of the method being at the present time in press.

Whether *Polyplax spinulosus* transmits the virus directly by its bite or by depositing the feces on abrasions of the skin has not been investigated. This question, incidentally, has not been solved satisfactorily for *Pediculus humanus*. Rats infested with lice show numerous superficial abrasions of the skin from scratches and bites. Another possible route of infection is the mucous membrane of the mouth. Infested rats are frequently busy in trying to bite the lice in order to remove them from the skin.

Our negative findings with fleas caught on the rats have little importance in view of the fact that the positive results of Dyer and his associates are, of course, of much more value than any number of mere negative experiments. Although we regard the rat louse as described above as the most likely vector of the disease from rat to rat, it has, of course, no importance in transmission of the disease from rat to man, since it does not feed on human beings. We regard fleas as the most likely transmitter of typhus from rat to man, particularly *Xenopsylla cheopis*, in view of the observations of Dyer and his associates, and of course, because of the fact that of the arthropods which are observed upon rats, they are the only ones which feed both on rats and on man. However, the problem still is fraught with some

uncertainty, in view of our work on ticks and bedbugs (5), and the brief survival of the virus in many insects must be taken into consideration.

CONCLUSIONS

Polyplax spinulosus, the common rat louse, is easily infected with the virus of typhus by feeding on infected rats. As in the case of *Pediculus humanus*, such feedings are followed by the appearance of large numbers of *Rickettsia prowazeki* within the gut of the insect. The virus of Mexican typhus can be transmitted from rat to rat by *Polyplax spinulosus* by methods of feeding simulating natural conditions. It seems, therefore, that this ectoparasite is an important factor in maintaining an endozoic of Mexican typhus among wild rats.

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STUDIES ON THE RELATIONSHIP OF CERTAIN VARIANTS OF *B. TYPHOSUS*

I. AGGLUTINATION AND AGGLUTININ ABSORPTION

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Serological analyses of the constitution of the motile bacilli have established the existence of a heat-labile agglutinin associated with the flagellae and of a heat-stable agglutinin contained in the bacillary bodies. Whereas there appears to be no antigenic difference in the flagellar substance of the smooth and the rough forms, the studies of the somatic antigens of these variants have not led to clean-cut results, although the tendency has been to consider them entirely dissimilar.

The purpose of the present paper is to present the results of cross-agglutination and agglutinin absorption tests, using the four known variants of *B. typhosus*, namely the motile smooth, non-motile smooth, motile rough, and non-motile rough forms, and to suggest a formula for the antigenic composition of these strains.

As a result of the work on the dissociation forms of various motile bacilli by Malvoz (1), Smith and Reagh (2), Orcutt (3), and Walker (4) dealing with the flagellar and somatic antigens and by Weil and Felix (5), Arkwright (6), Gardner (7), White (8), Li (9), and others working with the rough and smooth, as well as with the motile and non-motile forms, the following antigenic fractions have been assumed to be present in these organisms: (1) the flagellar antigen H; (2) the smooth body antigen O; (3) the rough body antigen ϕ or R. Thus, the smooth motile form (MS) would have the antigenic composition HO; the smooth non-motile form (NS) the composition O; the rough motile form (MR) the composition HO; and the rough non-motile form (NR) the composition ϕ . There would, therefore, be no somatic antigen common to the smooth and the rough variants.

The theoretical results of agglutination tests based on this assumption would be that the anti-MS serum would agglutinate all but the NR antigen and the anti-MR serum would agglutinate all but the NS antigen, while the anti-NS and anti-NR sera would agglutinate only the smooth and the rough strains respectively.

Li, working with these four variants of the hog cholera bacillus, has reported results in close agreement with this theoretical scheme of agglutination, except that his anti-NS (O) serum agglutinated the MR (HØ) antigen, although they presumably contain no common antigenic factor.

Gardner's actual experiments with *B. typhosus* do not correspond quite so closely with the theoretical results since his anti-MR serum agglutinated the NS culture and his anti-NR serum agglutinated both the MS and the NS cultures. These differences, however, he believes to be due to the fact that he used for the NS and NR antigens boiled suspensions of the corresponding motile forms and that the boiling not only destroyed the flagellar antigen but tended to change the smooth somatic (O) antigen to the rough (R) form.

In the course of a test to determine the immunological response to the usual typhoid vaccination with smooth and with rough vaccines, the writer (10) found that the sera of three individuals receiving the rough vaccine, containing both MR (HØ) and NR (Ø) forms, agglutinated an NS (O) culture, and that the sera of three individuals receiving the smooth vaccine, containing both MS (HO) and NS (O) forms, agglutinated an NR (Ø) culture.

Since these tests indicated that the smooth and rough variants of *B. typhosus* possess a common somatic agglutinin, a fact not in accord with the conception of antigenic composition outlined above, it was clearly necessary to confirm the results by agglutination and agglutinin absorption tests, using each of the four variants (MS, NS, MR, and NR) in pure culture.

EXPERIMENTAL

The variant strains used in the following experiment were derived from two cultures. The motile smooth strain (MS) and the non-motile smooth strain (NS) were obtained from motile and non-motile colonies of a culture freshly isolated from a case of typhoid fever. The motile rough strain (MR) and the non-motile rough strain (NR) were from a stock strain of *B. typhosus* (Rawlins). The following characteristics distinguished the four variants:

MS: Surface colonies on agar plate—smooth. Deep colonies in semisolid agar—large and irregular. Motile. Virulence for mice—0.01 cc. to 0.001 cc. of 18 hour broth culture. Number of organisms killed by 0.5 cc. of a 1/12 dilution of human blood—12 to 1,200.

NS: Surface colonies on agar plate—smooth. Deep colonies in semisolid agar—small and compact. Non-motile. Virulence for mice—0.01 cc. to 0.001 cc. of 18 hour broth culture. Number of organisms killed by 0.5 cc. of a 1/12 dilution of human blood—12 to 120.

MR: Surface colonies on agar plate—rough. Deep colonies in semisolid agar—large and irregular. Motile. Virulence for mice—1.0 cc. of an 18 hour broth culture. Number of organisms killed by 0.5 cc. of a 1/12 dilution of human blood—1,200,000.

NR: Surface colonies on agar plate—rough. Deep colonies in semisolid agar—small and compact. Non-motile. Virulence of mice—1.0 cc. + of an 18 hour broth culture. Number of organisms killed by 0.5 cc. of a 1/12 dilution of human blood—1,200,000.

The virulence of the NS culture for mice was usually somewhat greater, and never less than that of the MS culture. The NR culture was slightly less virulent than the MR culture.

In preparing the sera for the animal experiments, the rabbits were given seven intravenous injections of 0.5 cc. of the vaccines MS, NS, MR, and NR respectively at 7 day intervals, and were bled 9 days after the last injection. The antigens for the agglutination tests were the same suspensions that were used for producing the respective sera, but were diluted with three parts of broth for the agglutination tests.

The agglutinations were carried out by adding 0.5 cc. of a diluted formalinized broth culture to 0.5 cc. of the serum dilutions. The reactions were read after 2 hours in the water bath at 53°C. and again after 18 hours in the refrigerator. The suspensions used for absorbing the sera were 24 hour cultures on hormone agar in Kolle flasks washed off with normal saline, heated to 56°C. for 1 hour, divided into the requisite number of tubes, and centrifuged.

For absorption 2 cc. of a 1/10 dilution of the serum were added to the growth from one Kolle flask, incubated for 1 hour in the water bath at 53°C., centrifuged and the serum added to the growth from a second Kolle flask, returned to the water bath for an hour, and allowed to stand overnight in the refrigerator before centrifuging. Further absorption failed to reduce the titre of the sera.

The four antigens were controlled with normal rabbit serum, and showed no agglutination in dilutions of 1/100 and over.

From Table I, Column 2, it is seen that not only does the anti-MS serum agglutinate the NR culture and the anti-MR serum agglutinate the NS culture, but the anti-NS serum agglutinates the NR culture and the anti-NR serum agglutinates both the MS and NS cultures.

That the weak agglutination of the motile rough culture by the sera prepared with the non-motile antigens is not due to failure of antigen-antibody union is shown by the fact that the motile rough

TABLE I
Cross-Agglutination and Agglutinin Absorption Tests of Cultures MS, NS, MR, and NR with Anti-MS, Anti-NS, Anti-MR, and Anti-NR Sera

Table 1. Description Tests of Cultures MS, NS, MR, and NR with Anti-MS, Anti-NS, Anti-MR, and Anti-NR Sera						
1 Antigen	2 Serum unabsorbed	3	4	5	6	
		MS	NS	MR	NR	
Serum MS						
MS	12,800 F	6,400 G	12,800 F	6,400 G	6,400 F	
NS	6,400 G	0	0	0	0	
MR	1,600 F	0	1,600 F	0	1,600 F	
NR	3,200 G	0	0	0	0	
Serum NS						
MS	1,600 G	0	0	0	0	
NS	3,200 G	0	0	0	0	
MR	200 G	0	0	0	0	
NR	3,200 G	0	0	0	0	
Serum MR						
MS	12,800 F	6,400 G	12,800 F	6,400 G	6,400 F	
NS	3,200 G	0	0	0	0	
MR	6,400 F	0	6,400 F	0	6,400 F	
NR	3,200 G	0	0	0	0	
Serum NR						
MS	1,600 G	0	0	0	0	
NS	1,600 G	0	0	0	0	
MR	200 G	0	0	0	0	
NR	3,200 G	0	0	0	0	

F = flocculent agglutination.
G = granular agglutination.

F = flocculent agglutination.

G = granular agglutination.

O = no agglutination in a dilution of 1/100.

culture absorbs from these sera all the agglutinins for the three other cultures. 24 hour motile rough cultures agglutinate only slightly with the anti-NS and anti-NR sera, whether living, formalinized, or

killed at 56°C.; but 3 hour cultures agglutinate to titre whether living, formalinized, or heat-killed, as do the 24 hour cultures after heating to 75°C. for 30 minutes.

The cross-agglutination experiments here recorded were repeated on a number of occasions. 24 hour living broth cultures were substituted for the formalinized suspensions and sera prepared with boiled cultures of the motile strains were used in place of the anti-NS and anti-NR sera. The results, while varying slightly in agglutination titre, were always confirmatory of those given in Table I.

The absorption tests recorded in Table I show that whereas the non-motile cultures naturally fail to absorb the flagellar agglutinin from the anti-MS and anti-MR sera, each of the cultures completely removes the granular, somatic agglutinin from each of the sera, except that the MS culture still gives granular agglutination with the anti-MS and anti-MR sera after absorption with these cultures.

DISCUSSION

The cross-agglutination tests with the four variants of *B. typhosus* here reported can only be explained by assuming the presence of a common antigenic factor in the smooth and the rough forms, and the agglutinin absorption experiments confirm this assumption. The results reported by Li working with the same four variants of the hog cholera bacillus can readily be explained on the basis of the presence of three antigenic constituents, H, O, and Ø in different combinations in the four strains. Since the results here reported with *B. typhosus* cannot be explained on the basis of the presence of these three antigenic constituents, it would appear that one cannot generalize from the reactions of one member of the enteric group of organisms.

The possible objection that the cultures used in these experiments were not pure type variants was guarded against by numerous examinations of the cultures used in preparing the antigens. Both smooth and rough strains were examined on a number of agar plates, and the rough strains were further tested for the possibility of reversion to the smooth form by passage through mice and guinea pigs. Further, the rough strains were passed through twenty subcultures in 20 per cent homologous rough antiserum broth without increase in virulence or the appearance of smooth colonies. The non-motile strains were

examined microscopically and frequently inoculated into semisolid agar without showing any evidence of motility.

Although the results of the experiments here reported clearly point to the existence of a somatic agglutinin common to the four variants, there are two phases of the tests which require further discussion; first, the weak agglutination of the motile rough culture by the sera prepared with the non-motile antigens, and second, the persistence of the granular agglutination of the motile smooth culture by the anti-MS and anti-MR sera after absorption by the MS and MR antigens.

That the failure of the formalinized MR culture to be agglutinated by the anti-NS and anti-NR sera is due to a physical interference with the granular agglutination by the flagellae and not to a failure of the antigen-antibody union is shown by the fact that the MR culture absorbs from these sera the agglutinins for the other three variants, and by the fact that agglutination takes place if the flagellar agglutinin of the MR culture is destroyed by heating the antigen to 75°C. for $\frac{1}{2}$ hour. Furthermore 3 hour motile rough cultures, whether living, formalinized, or killed by heat, agglutinate to titre with the anti-NS and anti-NR sera. Although it is not clear why the young cultures agglutinate while the older cultures do not, it might be assumed that this condition is due to a relatively slow development of the flagellar antigen in the young cultures.

The persistence of the granular agglutination of the MS antigen by the anti-MS and anti-MR sera after absorption with the MS and MR cultures cannot well be explained as a non-specific effect since not only do these absorbed sera not agglutinate the MR antigen; but the MS antigen is not agglutinated by normal rabbit serum nor by anti-NS nor anti-NR serum when absorbed by any of the four variants. The possibility that the granular agglutination was due to insufficient absorption was guarded against by absorbing four times with a heavy suspension of MS followed by two absorptions with the dilute suspension used for antigen in the agglutination tests without reduction of the titre of the serum. Absorption with heavy suspensions of living MS culture likewise failed to remove the granular agglutinin, as did absorption with MS followed by absorption with NS. Therefore no explanation for the persistence of the granular agglutination in these cases has as yet been found.

To explain the results one must assume the presence of a common somatic antigen in the four variants of *B. typhosus* and of a flagellar antigen common to the two motile forms. Analyses, as yet incomplete, would indicate that the common somatic antigen is connected with the body protein of the bacilli, and that the factor which differentiates the smooth from the rough forms is the carbohydrate fraction which does not act as an agglutinin. Whether the difference between the smooth and the rough variants is due to the presence of two different carbohydrates, S and R, or to a difference in the amount of S in the two forms is as yet undetermined.

Since the initials H (*Hauch*) and O (*ohne Hauch*) first used by Weil and Felix to describe the appearance on agar of the motile and non-motile forms of *B. proteus*, have no significance when applied to *B. typhosus*, and since the designations O and Ø (or R) applied by other writers to the smooth and rough antigens do not appear, in the case of *B. typhosus*, to designate different substances but merely the same constituent combined in one case with the soluble specific substance (S) and in the other either with a small amount of S or with the carbohydrate of a rough form, the following fractions are suggested as composing at least the more important antigenic constituents of the four variants of *B. typhosus*: P, the somatic antigen common to all four forms; F, the flagellar antigen of the motile forms; S, the soluble specific substance contained in the smooth forms; and R, the rough carbohydrate. The antigenic composition of the variants would then be as follows:

Motile smooth (MS)	= P + S + F
Non-motile smooth (NS)	= P + S
Motile rough (MR)	= P + F (+R)
Non-motile rough (NR)	= P (+R)

In this theoretical composition, the experiments here described establish the presence of the same somatic agglutinin (P) in all four variants and give no experimental support to the previous theory that the smooth virulent organisms had an antigen O distinct from the substance Ø of the rough non-virulent organisms. The existence in the motile forms of a heat-labile flagellar agglutinin F is confirmed; but the nature of the substance S to which the virulence of smooth

forms is due is merely suggested as analogous to the soluble specific substance of the pneumococcus. Preliminary tests have shown the presence of one or more carbohydrates in approximately equal concentration in the two forms; but whether there is a distinct carbohydrate R in the rough organisms or whether there is another carbohydrate present in both the "S" and "R" forms and not associated with virulence, similar to the "C" substance of the pneumococcus, described by Tillett, Goebel, and Avery (11) remains to be determined, since these fractions do not appear to enter into the agglutination reactions.

CONCLUSIONS

The results of cross-agglutination and agglutinin absorption experiments with the motile smooth, non-motile smooth, motile rough, and non-motile rough forms of *B. typhosus* are presented.

Cross-agglutination between these four forms is complete, save that the motile rough antigen is under certain conditions only weakly agglutinated by the antisera prepared with the non-motile forms.

Cross-absorption of the somatic agglutinin of the four variants is complete, save that the motile smooth culture still shows granular agglutination with the anti-MS and anti-MR sera after absorption with these cultures.

A theory of the antigenic composition of the four variants of *B. typhosus* is presented, based on the results obtained in these experiments.

It would appear that, contrary to the usually accepted theory, the four variants have a common somatic agglutinin. To explain the difference between the smooth virulent forms and the rough non-virulent forms it has been assumed that the S forms contain a carbohydrate which is associated with virulence and which takes no part in the agglutination reaction.

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CUTANEOUS REACTIONS IN RABBITS TO THE TYPE-SPECIFIC CAPSULAR POLYSACCHARIDES OF PNEUMOCOCCUS

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It has previously been shown (1) that, following the intradermal injection of the type-specific polysaccharides of *Pneumococcus*, there develops in the skin of patients convalescent from pneumonia an immediate wheal and erythema reaction to the polysaccharide homologous in type to that of the pneumococcus causing the infection. The reaction is type-specific and its occurrence is always associated with the presence in the patient's serum of specific antibodies for the homologous type of *Pneumococcus*.

Since the purified carbohydrates are capable of eliciting this striking response in the skin of human individuals who, upon recovery from lobar pneumonia become spontaneously immune, it seemed desirable to study the cutaneous reactivity of experimental animals which had been artificially immunized to type-specific pneumococci. The purpose of this paper, therefore, is to report the occurrence of a local cutaneous reaction to the type-specific polysaccharides in rabbits actively or passively immunized to the pneumococcus.

Materials and Methods

1. Immunization of Rabbits.—

(a) *Active Immunization.*—Groups of rabbits were immunized with pneumococci of Types I, II or III. Other animals were immunized with a non-type-specific R strain of pneumococcus. Immunization was carried out by the method of Cole and Moore (2). 10 days after the final injection, blood was obtained from an ear vein and the serum tested for the presence of type-specific antibodies.

(b) *Passive Immunization.*—Antipneumococcus rabbit serum was obtained from animals immunized as above. Antipneumococcus horse serum for each of Types I, II and III was obtained from the New York State Board of Health. Rabbits were passively immunized by the intravenous injection of 5 to 15 cc. of

one or other of these sera. The following day blood was obtained from an ear vein for the determination of circulating type-specific antibodies.

2. Determination of Type-Specific Antibodies.—

(a) Type-specific antibodies were detected by the precipitin test; 0.2 cc. of serum diluted to 0.5 cc. with physiological saline was mixed with 0.5 cc. of varying dilutions of the type-specific polysaccharides. Readings were made after 2 hours in the water bath at 37°C. and overnight in the ice box.

(b) Type-specific agglutinins were determined by the usual method using dilutions of serum and suspensions of heat-killed organisms.

*3. Skin Testing Materials.—*Skin tests were made with the capsular polysaccharides of pneumococcus derived in purified form from *Pneumococcus* Types I, II and III. These substances are chemically and serologically distinct. They are protein-free. The Type II and Type II polysaccharides are also nitrogen-free; the Type I material contains 5 per cent non-protein nitrogen which is part of the sugar molecule (3). The polysaccharides were dissolved in physiological saline in a concentration of 1:200 and 0.1 cc. of this solution, containing 0.5 mg. of polysaccharide, was used for each injection. The animals were closely observed for the occurrence of an immediate or delayed reaction. Measurements were recorded 24 hours following the injection and daily thereafter.

In most instances the specific capsular polysaccharides of Types I, II and III *Pneumococcus* were injected simultaneously into neighboring sites in the rabbit's skin.

Skin Reactions in Actively Immunized Rabbits

Three series of rabbits were actively immunized with pneumococci of Types I, II and III, respectively. After immunization, injections of the type-specific polysaccharides into the skin were made.

Protocols of rabbits representative of the three different immune groups illustrate the conditions of experiment and characteristic results.

Rabbit 4-74 was actively immunized to Type I *Pneumococcus* by the intravenous route. 10 days after the final immunizing injection, the animal's serum contained specific precipitins for the Type I polysaccharide in a titer of 2,560,000. The following day injections of 0.5 mg. of each of Types I, II and III polysaccharides were made into the shaved skin of the side. 2½ hours later a few faint purpuric spots were observed at the site of injection of the Type I material. The lesion increased in size until at 24 hours a purpuric edematous area 2.5 cm. in diameter was noted. In 48 hours the diameter had reached 3.0 cm., the edema was less but the purpura had darkened and infiltration had increased. A gradual clearing took place over the next 4 days. At the sites of injection of the Type II and Type III materials no evidence of reaction was observed.

Rabbit 4-41 was actively immunized against Type II *Pneumococcus*. 10 days after immunization was completed the serum contained precipitins for the Type II polysaccharide in a titer of 1,280,000. The following day, injections of 0.5 mg. of each of the Type I, II and III polysaccharides were made into the skin of the side. 24 hours later, at the site of injection of the Type II polysaccharide, a firm edematous lesion, bright red in color, and measuring 4.5 cm. x 2.3 cm. in cross diameters was noted. No purpura was present. The lesion had practically cleared in 4 days. No reaction was elicited by the carbohydrates of Types I and III.

Rabbit 14-85 was immunized with a strain of Type III *Pneumococcus* which had, by repeated animal passage, acquired virulence for rabbits. On the 10th day after the last immunizing dose the serum contained precipitins for the Type III polysaccharide. With a 1:80,000 dilution of the polysaccharide a ++++ reac-

TABLE I

Skin Reactions to Type-Specific Polysaccharides in Rabbits Actively Immunized with Pneumococci of Homologous Types

Type of <i>Pneumococcus</i> used for immunization	Type-specific precipitins present		No type-specific precipitins present	
	No. of rabbits	No. giving skin reaction	No. of rabbits	No. giving skin reaction
I	11	10	4	0
II	9	8	3	0
III	5	3	8	0
R	—	—	5	0

tion was obtained. Higher dilutions were not tested. The following day 0.5 mg. of each of the polysaccharides of Types I, II and III was injected into the shaved skin of the side. After 24 hours at the site of injection of the Type III polysaccharide, there was noted a slightly elevated, firm, purpuric area 2.6 x 3.4 cm. in diameter, surrounded by a white zone of edema. The lesion gradually subsided until the 6th day when only a faint pigmentation remained. The reaction was type-specific; no reaction was produced by the Types I and II carbohydrates.

The foregoing protocols demonstrate clearly the form of reaction obtained and the conditions under which it was produced. Table I shows the frequency with which skin reactions were elicited by the intradermal injection of the homologous polysaccharide in rabbits actively immunized with pneumococci of Types I, II or III, or with an R strain of pneumococcus.

It is seen in Table I that of twenty-five animals yielding serum in which type-specific precipitins were present, all but four gave a typical local inflammatory reaction to the injection of the carbohydrate of the homologous type. In these four instances the titer of antibodies was lower than in the reactive animals. On the other hand, those animals in which circulating type-specific precipitins could not be demonstrated failed in every instance to give a positive response. No reaction was obtained in any case except to the homologous type-specific polysaccharide.

In contrast to the immediate wheal and erythema reaction produced by the carbohydrates in patients convalescent from lobar pneumonia, the reaction produced in the artificially immunized rabbits is usually first detected after 6 to 8 hours. The reaction makes its appearance as a faint blush and gradually increases in size, producing a pink edematous area frequently surrounded by a zone of pale edema. The edema tends to increase and erythema is replaced by a purplish discoloration. The lesion reaches its height in 24 to 48 hours and at that time it may be 4 to 5 cm. in diameter and 0.5 to 1.0 cm. in elevation. A gradual regression takes place in 4 to 7 days until little or no trace of the reaction remains. With the doses employed necrosis of the skin has not been observed. The cutaneous reaction was produced in rabbits only by the polysaccharide homologous in type to that of the pneumococcus employed in the process of immunization. The severity of reaction appears to be related to the titer of type-specific precipitins for the homologous polysaccharide in the serum of the animal. In certain instances, despite the presence of type-specific precipitins, no cutaneous response was evoked by the carbohydrate, but in no case was a reaction produced in the absence of circulating type-specific precipitins.

Skin Reactions in Passively Immunized Rabbits

Attempts were made to transfer reactivity, passively, to normal rabbits by the intravenous injection of serum from immune rabbits known to give positive skin tests and the serum of which contained a high titer of type-specific precipitins. 5 to 40 cc. of serum were injected. In none of the recipient animals, however, was a positive skin reaction induced, nor were precipitins demonstrable in their sera.

The failure in these instances may be due to the fact that insufficient immune serum was injected.

Attempts were next made to induce skin reactions to the type-specific polysaccharides in animals passively immunized with serum of horses immune to *Pneumococcus* Types I, II or III. Fourteen rabbits were injected: five with Type I serum, three with Type II serum and six with Type III serum. 10 to 15 cc. of immune serum were injected intravenously, although in one rabbit only 5 cc. of serum were used. The following day the serum of all the passively immunized rabbits was found to contain a high titer of precipitins for the homologous polysaccharide and in each instance a well marked cutaneous response to the intradermal injection of the polysaccharide occurred. The character of the reaction was identical in every respect with that obtained in actively immunized animals. No reactions to the polysaccharides of heterologous types were observed.

Local passive transfer of sensitivity was carried out in rabbits by infiltrating each of two areas of skin with 1.0 cc. of immune horse serum. After 24 hours there were seen at the sites of serum infiltration small flat areas of erythema. Into one of these areas 0.5 mg. of the homologous polysaccharide was injected and into the other, as control, an equal amount of a heterologous polysaccharide. 24 hours later, at the site of inoculation of the homologous polysaccharide, a deep red, elevated edematous area 2 to 4 cm. in diameter was observed and this persisted for 48 to 72 hours. At the control site there remained only a pale erythema due to infiltration of the serum.

Guinea pigs were passively immunized by intraperitoneal injections of antipneumococcus horse or rabbit serum. The animals which received immune horse serum showed no signs of local or general reaction to the subsequent intradermal injection of the homologous type-specific polysaccharide. Similarly, no cutaneous reaction to the polysaccharide was observed in those guinea pigs passively immunized with rabbit serum. In several of the latter group, however, mild signs of general anaphylaxis (restlessness, coughing, scratching of nose, etc.) were noted shortly after the introduction of the specific substance into the skin. Tillett and Avery (4) were able to induce fatal anaphylactic shock with minute amounts of the type-specific polysaccharide in guinea pigs passively sensitized with homologous

antipneumococcus rabbit serum. If horse serum was employed for sensitization no response occurred. In the present instances the observation that anaphylactic symptoms were produced in guinea pigs receiving antipneumococcus rabbit serum but not in those prepared with immune horse serum, appears to be another demonstration of the facts reported by these workers.

DISCUSSION

The results reported in the present paper demonstrate that in rabbits actively or passively immunized to a type-specific pneumococcus the intradermal injection of specific polysaccharide of homologous type elicits a definite form of cutaneous reaction. In contrast to the immediate wheal and erythema reaction observed in humans convalescent from pneumonia, the reaction produced in the immune rabbit is delayed and resembles in its general appearance the form of reaction first described by Arthus (5). The reaction is characterized by slow development, edema, erythema and purpuric discoloration. The capacity of the individual rabbit to give a positive skin reaction is closely associated with the presence of demonstrable type-specific precipitins in its serum. In no instance was a positive reaction observed in the absence of demonstrable type-specific precipitins. In the guinea pig no local skin reaction was elicited but signs of general anaphylaxis were observed.

It is plain that one can correlate the reactions induced in immunized individuals of three species (man, rabbit and guinea pig) by the intradermal injection of a protein-free carbohydrate of bacterial origin. The result is the same, apparently, whether the immunity has been actively or passively acquired. In all three species the form of response to carbohydrate is typical of the reactions commonly considered to be of an anaphylactic nature when produced by protein materials.

The reactions are elicited only in the presence of specific antibodies for the capsular polysaccharide of *Pneumococcus* and then only by the specific polysaccharide homologous in type to that of the pneumococcus inducing the antibody response. Julianelle has shown (6) that animals which have received repeated intravenous injections of unencapsulated R forms of *Pneumococcus*, or of the nucleoprotein

TABLE II
Relation of Hypersensitiveness and Immunity to Pneumococcus in Rabbits

Material used for immunization	Route of immunization	Antibodies produced		Immunity against virulent pneumococci		Skin reaction to pneumococcus protein	Skin reaction to capsular polysaccharide	Summary
		Species-specific	Type-specific	Active resistance	Ability of serum to protect mice			
Pneumococcus nucleoprotein	i. v. or i. c.	+	-	-	-	+	-	Hypersensitiveness to pneumococcus protein but no immunity to pneumococcus infection
R form of pneumococcus	i. v. or i. c.	+	-	+	-	+	-	Broad increased active resistance to all types of Pneumococcus with protein hypersensitiveness
S Pneumococcus	i. c.	+	Present in low titer in only 12%	+	Protection against high dilutions in only 21%	+	-	Same as above
S Pneumococcus	i. v.	+	+	+	+	+	+	Broad active anti-R resistance. Anti-S type-specific immunity. Hypersensitiveness to protein. Reaction to polysaccharide

of the pneumococcus, or have received intracutaneous injections of unencapsulated or even of encapsulated organisms develop only species-specific, antismatic antibodies and a hypersensitiveness for the proteins of the pneumococcus. Type-specific antibodies for the capsular polysaccharide could not be detected in the serum of animals so treated, nor were any skin reactions produced by the injection of type-specific polysaccharides. Similarly, in the present experiments no reactions to the carbohydrates were obtained in animals immunized to the R organisms nor in fact, in any animal the serum of which contained no type-specific precipitins. Furthermore, in man, the reaction obtained with nucleoprotein does not take the form of a wheal and erythema, but is a delayed reaction reaching its height in 18 to 24 hours. It is evident, therefore, that the species-specific antismatic, or antiprotein, antibodies play no rôle in the production of the skin reactions to the capsular polysaccharides. Moreover, the data presented stress again the sharp type specificity of all reactions involving the pneumococcus capsular polysaccharides.

The purpose of this report is not to enter into a discussion of the relation between hypersensitiveness and immunity but a few noteworthy facts may be mentioned. These are presented in tabular form in Table II. Hypersensitivity to the protein of the pneumococcus can be induced in rabbits by repeated injections of nucleoprotein. Under these conditions, antibodies for the species-specific R Pneumococcus develop but neither active immunity nor any capacity of the serum to confer passive immunity upon another species can be demonstrated (7, 8). In this case, hypersensitivity to the bacterial protein is not associated with increased resistance to pneumococcus infection.

If an R form of *Pneumococcus* is employed for immunization, only species-specific (anti-R) antibodies appear in the serum of the animal. Protein hypersensitivity can be demonstrated in this animal but no reaction is elicited by any of the capsular polysaccharides (6). In addition, the animal can be shown to have acquired an increased resistance to virulent pneumococci of Types I, II and III (8). Hence, this resistance is not type-specific. The height of the immunity is not great and is passively transferable only with great difficulty within the same species. The serum of such an animal does not confer passive protection upon mice. Here, then, is an example of protein

hypersensitiveness associated with a broad, non-type-specific increased active resistance.

When an animal has been effectively immunized with a virulent, encapsulated type-specific pneumococcus, type-specific anticapsular antibodies appear in the circulating blood. The animal has developed not only a highly efficient type-specific active immunity but its serum affords passive protection to mice against many lethal doses of pneumococci of the same type. This type-specific immunity is the form observed in convalescents from lobar pneumonia. In association with immunity due to type-specific antibodies, the intradermal injection of the type-specific capsular polysaccharide incites a response characteristic for the species. The reaction to the intradermal injection of the capsular polysaccharides of *Pneumococcus* may be termed, therefore, a hypersensitive or anaphylactic response occurring only in the presence of type-specific immunity.

SUMMARY

The injection of the type-specific capsular polysaccharides of *Pneumococcus* Types I, II and III into the skin of rabbits, actively or passively immunized to one of these types of *Pneumococcus*, elicits a type-specific cutaneous reaction. The form of reaction resembles that described by Arthus. The reaction is produced only when type-specific precipitins for the homologous polysaccharide are demonstrable in the blood of the rabbit. In 84 per cent of actively immunized rabbits, the serum of which contained type-specific precipitins, a reaction was elicited. A positive result was obtained in 100 per cent of rabbits passively immunized with antipneumococcus horse serum whereas, attempts passively to transfer reactivity from immune rabbit to normal rabbit were unsuccessful. The recipients, in the latter group, possessed no demonstrable circulating type-specific precipitins. The reaction produced by specific capsular carbohydrates is always associated with a well grounded type-specific immunity.

A brief summary of the relation of hypersensitiveness and immunity to pneumococcus is given.

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SERUM SICKNESS IN RABBITS

I. MANIFESTATIONS OF SERUM SICKNESS

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PLATE 54

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The occurrence in man of reactions subsequent to a first injection of certain foreign proteins has long been recognized and, following the general use of antisera of various types, these reactions were thoroughly studied by von Pirquet and Schick (1) who gave the name serum sickness or serum disease to this phenomenon. It was possible to study it quite extensively as it occurs in man. However, the occurrence or production of an analogous condition in laboratory animals has not to our knowledge been reported and it has, therefore, been impossible to study this condition experimentally.¹

It appeared improbable that man should be the only species in which serum sickness might occur and our effort to produce this disease was based upon the assumption that some laboratory animals should be susceptible under certain conditions. The literature concerning serum sickness in man shows quite definitely that the occurrence rate of this disease is greater in proportion to the amount of antiserum injected. It seemed, therefore, that experimental work would have to be carried out with injections of relatively large doses of serum. The animals used in these experiments, rabbits, were chosen because in some respects at least they seem to stand closer to man in reactions of hypersensitiveness than do guinea pigs.

¹ Bédère, Chambon and Ménard (2) noted in cattle the appearance of varied forms of eruptions, rise of body temperature and evidences of disability associated with locomotion appearing approximately 4 days after the injection of large quantities of horse serum.

It was, of course, impossible to assume in advance what phenomena might be expected as evidences of serum sickness in the rabbit following the administration of a single large dose of horse serum. The animals were, therefore, kept under close observation subsequent to injection. They were observed as a routine at about 9 a.m. and about 4 p.m.; particularly in the earlier experiments several observations were made at night during the fifth, sixth, seventh and eighth 24 hour period. It became evident, however, that the morning and afternoon observations were adequate for noting the important reactions. Fortunately the reaction was quite an obvious one. At about the 5th or 6th day after injection of a single large dose of normal horse serum, it was evident in some cases that the rabbit's ears were distinctly reddened. On closer examination there was an erythema involving a large portion of the ear, especially intense in the basal portion but involving the lower two-thirds. In addition, pressure made evident that this erythema was accompanied by edema which varied considerably in degree but which also involved all or part of the lower two-thirds of the ear.

Our first successful results were obtained with animals which had received 5 cc. pro kilo.

Previously and also later, we injected animals with 3 cc. pro kilo but in no case was a positive result obtained; only a total of eight rabbits received this smaller dose but in view of the per cent occurrence when we used larger doses we should have expected at least one to five of these animals to give evidence of a reaction. Only one animal showed a minimal and limited erythema on the 8th day after injection; this erythema might be considered as suggestive but the reaction was rather of the type which we have not accepted as being actually positive in other groups of animals. We are inclined to believe, therefore, that an injection of 3 cc. pro kilo is too small a dose to produce reactions and that in all probability 5 cc. pro kilo are practically the minimal amounts required to cause the appearance of this phenomenon in rabbits. We also increased the amounts injected, using 7.5 cc., 8 cc. or 10 cc. pro kilo with essentially the same results as when the 5 cc. doses were injected; the comparative results using the various quantities are analyzed below.

The serum in our first experiments was injected into the marginal vein of the ear. Since the reactions were evident on the ears the question naturally arose as to whether they were not dependent upon extravasation of serum into the tissues of the ear or possibly to

trauma associated with the manipulation of the ear. It appeared that this was not the case in view of the fact that the reactions occurred both in the ear used in the injection and in the other ear. However, we used other routes for introduction of the serum; at first, because the reactions had followed intravenous injection, we injected a number of animals either intracardially or intrajugularly (this latter method necessitating operative procedure), and it became evident in view of the positive results in these latter groups that the site of intravenous injection had no influence on the result. Rabbits were also injected intramuscularly and a few subcutaneously and in these also the reactions appeared in the ears. In the majority of later experiments the serum was injected into the subscapular tissues² since this route of administration was essentially one of the easiest and since the results compared favorably with any of the other modes of administration which were tried. The serum was in each animal injected half under the right scapula and half under the left one.

In the majority of our experiments we used white rabbits since they proved to be more satisfactory than colored ones. The reactions were more evident on their ears than on the ears of rabbits having darker hair. However, marked reactions were observed in brown and even black haired rabbits and there was no difficulty in recognizing the reactions in these animals. Our impression however was that the occurrence of the reaction was somewhat less frequent in the colored animals, but the number of these does not permit a final statement. It is possible also that certain conditions associated with the experiments carried out with these colored animals were responsible for the smaller percentage of positive reactions.

It may be noted that the occurrence of hemorrhages, localized erythema and localized edema is more frequently seen in ears of normal white rabbits than in the ears of normal colored animals.

As previously stated the reactions were quite evident but they must and can readily be differentiated from the reactions occurring in the normal rabbit. The anatomical distribution, apart from the intensity

² This route of administration was suggested by Dr. John Auer on the assumption that the massaging due to the constant movement of the forelegs would assist in more rapid absorption such as might take place if the serum were injected intramuscularly.

and extent of the reactions, was a cardinal point in this connection; it therefore becomes necessary to consider various areas of the rabbit's ear.

It is preferable to examine the ear by daylight and by both diffuse reflected light and transmitted light (preferably therefore in a well lighted room with the observer facing the window). Artificial light can be used but when viewing the ear by transmitted light, the source must not be too bright; indirect reflection of the artificial light is preferable. Direct sunlight is not satisfactory since the intense light gives a pink color to the normal ear due to the light passing through the vascular bed and the cartilage. It is of advantage to examine both the posterior and anterior aspect of the ear.

Roughly one may divide the ear into two vertical divisions lying external and internal to the central vessels and horizontally into thirds which are spoken of as lower, middle and upper; occasionally the division may be made on the basis of quarters.

One area of the ear must be especially noted; this is an area which for the sake of a descriptive term will be referred to as the triangle. Actually this area may include nearly all of the middle third and possibly a portion of the upper third of the external half of the ear; it is referred to as a triangle however because reactions here tend to have a somewhat triangular form with the base toward the marginal vein and the apex pointing downward and inward towards the central vessels. This area usually appears more vascularized than other portions of the ear.

Attention may also be called to the frequent presence of a cartilaginous fold well towards the base of the ear and several millimeters (3 to 7) inside the external margin of the ear.

We have observed the ears of 147 normal rabbits for periods varying from 2 to 7 weeks in order to determine what reactions occur under normal conditions. One observation a day was made upon these animals. The normal animals were kept under various conditions; from six to twelve together in runways or one to three rabbits in smaller cages. The reactions in normal rabbits do not depend upon factors influenced by manner of caging: all types of reactions were observed regardless of the manner of housing the animals.

Since certain reactions did appear in normal animals the possibility of food influencing the occurrence of these was studied. Normal animals were fed on oats, hay, (alfalfa or prairie alternating) and vegetables (carrots and cabbage); or this same diet with the addition of a commercial rabbit "chow;" or third, on oats, "chow" and vegetables. Regardless of the diet the reactions were essentially the same.

Hemorrhages, fairly extensive or petechial in character, are frequently, in fact

almost constantly, noted on the ears of normal rabbits. They may appear in almost any portion of the ears. Frequently they are associated with trauma such as scratches either on the posterior or more frequently on the anterior surface of the ear. At times the hemorrhagic areas suggest definitely by their arrangement that they are due to scratches but it may be impossible to observe any abrasions associated with these. It may be noted that hemorrhages of varying degree appear both in rabbits which were caged with others and in those which were alone in a cage. Hemorrhages in the ear may at times result in thickening of the ear at such areas. Hemorrhages are to be considered as normal appearances and not in any way characteristic of the reactions associated with serum sickness in rabbits.

Definite vascular changes of various types may be noted. A dilatation of the larger vessels occurs more or less frequently; somewhat less frequent in occurrence is a widespread dilatation of the entire capillary (or arteriolar and venular) bed of the ear. Particularly this latter type of appearance may rise and subside as the ears are watched for a minute or more. These reactions are to be considered essentially as of normal occurrence. However, it has frequently been noted that in injected animals from 6 to 24 hours before the appearance of the typical reactions the alternation of dilatation and contraction of the small vessels may be seen; this is not a constant occurrence however. It must be recognized that while these reactions of vascular instability may give to the ears the appearance of marked redness, this appearance comes and goes as one observes the ears, and has no permanency.

Often about the larger vessels and less frequently about the smaller ones a slight redness extending for 1 or 2 mm. on either side may be noted, giving the vessels a fuzzy appearance. This is not due to hemorrhage since it disappears on pressure. This reaction like those discussed above is to be considered as one occurring in animals which had not received horse serum.

Areas of duskiness may often be noted on the ears. A limited area may appear darker than the remaining ear surface; most frequently this occurs in the outer median area (triangle) of the ear. Frequently it appears associated with the fading of scattered petechial hemorrhages in this portion, and may then have a brownish dusky appearance. These reactions again are essentially different from those appearing in the ear after injection of serum.

Limited and scattered areas of erythema appear from time to time in a very large per cent of the normal rabbits examined. These areas are usually discrete and relatively small and may occur anywhere in the upper two-thirds of the ear, again more often in the limited portion within the middle outer third (triangle) of the ear. Frequently also such areas are seen well towards the median margin in the upper two-thirds of the ear. Occasionally such areas are noted in the lower portion but it must be kept in mind that in the normal animal they involve only limited areas and do not simulate the widespread erythema seen in injected animals. Most frequently one small erythematous area from 0.5 to 1 cm. in

diameter may be seen; less frequently but by no means exceptionally two or three and occasionally more such areas may be noted but they are usually quite widely separated. In only three of the 147 normal rabbits was a widespread distribution of the erythema noted; one of these cases was associated with petechial hemorrhages involving a large part of the ear; in the other two the erythema involved chiefly the two portions mentioned above—the outer middle third and the inner upper two-thirds of the ear. It is essential in examining the ears that the presence of scratches associated with the erythema be carefully searched for since frequently these are the presumptive causal factors underlying the limited erythematous areas.

Edema may at times occur associated with the erythematous or hemorrhagic areas. Actually this has been noted at one time or other in 35 of the 147 normal animals, that is in approximately 23 per cent of animals. It must be clear from what has been said regarding the location of the erythematous areas that edema since it usually occurs with erythema is noted chiefly in the upper two-thirds of the ear, or in the so called triangle or along the inner margin. Occasionally edema

TABLE I
Degree of Edema in Normal Rabbits' Ears

Edema.....	?	±	+	++
No. of rabbits.....	2	16	12	5

is also noted along the line of the median vessels. In only three normal animals has edema been noted in the lower third of the ear. The presence of the edema is usually determined by pressing the ear between the fingers and then palpating for the ridge which should be present. The degree of edema varies widely; at times the ridge about the pitted area is just palpable, or at the other extreme the pitting resulting from the pressure may be visible to the eye. We have attempted to express the degree of edema by a scale running from very slight to marked, using as symbols; ?, ±, + or ++. The occurrence of various degrees of edema as noted in the ears of normal rabbits appears in Table I. It is doubtful, however, whether the degree of edema is of any importance in differentiating between the reactions in normal and in injected animals. It is rather the location and the associated reaction which set apart the reaction in the injected animals.

After the above description of the reactions which may be noted in normal rabbits it is possible rather briefly to point out the characteristics of the reactions occurring in injected animals. Only two of the above mentioned phenomena are to be considered as characterizing the reaction of serum sickness in the rabbit; these are erythema and edema. The general picture of the reactions in the injected animal is

sharply different from that seen in the normal animal, and a few observations of the positive reactions actually serve best to fix their distinctive appearance.

In all 136 injected animals are included in the observations here discussed; 103 of these were injected with normal sera from various horses, while 33 were injected with various antisera.

While the erythematous reactions in normal animals are discrete and limited in the areas involved, in the injected animal the greater part of the ear is reddened and is permanently red (that is we are not dealing with a transient vascular or capillary flushing). There is more or less of a pattern in the involvement; the central upper portion of the ear is more likely to be uninvolved leaving a wedge-shaped area relatively white and pale; at times the point of the wedge extends downward along either or both sides of the central vessels, reaching occasionally to the lower third of the ear. The outer upper quarter

TABLE II

Degree of Edema in Ears of Rabbits Subsequent to Injection of Serum

Edema.....	?	±	+	++
No. of rabbits.....	7	11	29	17

may also be unaffected. The character of the erythema is different from that seen in normal animals. It is diffuse, not discrete, often fairly uniform in intensity or at times darker patches appear against the lighter flushed background. Often the erythematous ears appear distinctly mottled. The intensity varies and one may see reactions varying from a definite pink to an intense red. At times the erythema is morbilliform or at others it may be scarlatinal. Usually both ears of the rabbits are affected but occasionally only one ear shows the erythema; it is, however, exceptional that erythema occurs in only one ear.

Erythema is more frequently noted than is the edema, and it may occur without the presence of edema. The edema may affect only one ear although the involvement of both ears is frequent. The edema associated with the reaction in rabbits varies in degree much as does the edema in normal animals. In Table II we have shown the number

of rabbits showing varying degrees of edema subsequent to injection of serum and have noted in each animal only the most marked degree of edema. It can be seen that there is a slight tendency for more animals to show marked reactions in the injected group than in the normal animals. However the intensity of the reaction is in no way to be considered as differentiating the typical reactions in injected animals. The location and extent of the edema are the characterizing qualities. Edema occurs in serum sickness in rabbits in the lower third of the ear which portion as noted above is rarely affected in the normal animal; it may appear either lateral or medial to the central vessel or on both sides, or occasionally it affects the small area outside the cartilaginous thickening mentioned above. The edema often involves not only the lower portion of the ear but extends upwards and affects the entire lower half or two-thirds of the ear; in such cases there is a tendency for the edema to be more marked in the basal portion and to become less intense in the upper portion. Almost without exception the upper third or quarter has been found free of edema. Both ears may be affected or only one and it is not infrequent to find only a limited basal area on one ear edematous. The edema is then far less constant in occurrence and less widespread in distribution than the characteristic erythema. In a number of animals the edema may appear and disappear only to reappear on a subsequent day. More frequently however the edema after once diminishing does not reappear or increase in intensity. Usually the edema is associated with the erythema, that is, they are both present; as stated above however erythema may appear without edema, and in a few cases (three) edema persisted even though the general erythema had disappeared. In one animal only did basal edema appear without previous or subsequent generalized erythema.

It is evident then that the reactions subsequent to serum injections were strikingly different from those seen in normal animals and these latter cannot be mistaken for the typical reactions. It is probable that mild reactions not as definitely characteristic as those just described above occur in injected rabbits, manifested usually by irregular and relatively limited erythematous reactions on the ears, since frequently about the 5th, 6th or 7th day after injection such evidences were noted; we have, however, not listed such doubtful reactions as

positive. In all about thirteen animals in our series showed such doubtful reactions but in order to remain on a safe basis we have considered animals presenting such reactions as non-reacting. It seemed to us preferable to set a distinctive and definite picture to be considered as the positive reaction.

The number of animals observed is apparently adequate to serve for statistical presentation and therefore there are given below tabulations of various observations relating to the reactions in the rabbits. In this analysis only the rabbits injected with normal horse serum have been considered since it appears advisable for various reasons to consider separately at some later time the group of animals injected with the antisera. It is evident from Table III that approximately

TABLE III

Occurrence of Various Types of Reactions in Ears of 103 Rabbits Subsequent to Injection of Horse Serum

General erythema and edema, both definite	General erythema, positive; edema questionable	General erythema, positive	Edema, positive	Total considered positive
58	5	7	1	71 (68.9%)
General erythema, questionable	Edema, questionable	No reaction		Total considered negative
7	6	19		32 (31.1%)

70 per cent of the injected animals showed what we have considered a positive reaction. The percentage occurrence of serum sickness in this series of animals compares relatively well with the percentage reported as occurring in man following the injection of large doses of serum.

Since the sex was not noted in ten of the animals used in some earlier experiments the number of animals in Table IV showing the occurrence of reactions in the two sexes is smaller than the total used and listed in Table III. While it appears from Table IV that the reactions show a lower incidence in females than in males we hesitate to assume that this would be a correct statement of the fact. We have noted frequently that pregnancy seems to cause a suppression of the reaction and a number of the female rabbits proved to have been pregnant

during the period of the experiment. We may therefore possibly assume that existence of pregnancy may have been a factor in the lower occurrence of reactions in the females.

The body weight of the animals had apparently no influence upon the occurrence of the reactions as shown in Table V. We may use

TABLE IV
Occurrence of Reactions in Ears of Rabbits of Various Sex

	General erythema and edema, both definite	General erythema, positive; edema questionable	General erythema, positive	Edema, positive	Total positive	Total considered negative
No. of males.....	30	3	2	0	35	10
No. of females.....	24	1	1	1	27	20

TABLE V
Occurrence of Reactions in Rabbits' Ears in Relation to Body Weight

	1600-2000 gm.	2001-2500 gm.	2501-3000 gm.	3001 gm. upwards
Total.....	12	36	25	14
No. positive.....	6	29	15	10
No. negative.....	6	7	10	4

TABLE VI
Occurrence of Reaction in Rabbits' Ears in Relation to Route of Injection of Serum

	Ear vein	Intra-cardial	Intra-jugular	Intra-muscular	Subcutaneous	Into sub-scapular region
Total.....	33	6	13	4	5	42
No. positive.....	25	4	8	4	2	28
No. negative.....	8	2	5	0	3	14

the weight as a rough measure of age and, therefore, age apparently has no bearing on the occurrence of the reaction. The only group in which the percentage occurrence is distinctly lower is in the smaller or younger animals but it is doubtful whether the numbers used in the group justify a final conclusion.

The influence of the route of introduction of the serum is shown in

Table VI. The figures are presented regardless of the quantities of serum injected. Irrespective of the route of administration of the serum, positive reactions were obtained. It is also evident that the reactions on the ears were not in any way dependent upon extravasation of horse serum into the tissues of the ear during the course of injection, which might have been the case when injections were made into the ear vein. Certainly introduction of the serum by any of the routes used, excepting through the ear vein, should result in no abnormal or special localization of serum in the tissues which later showed the reaction. In the small series of animals given subcutaneous injections the percentage occurrence of positive reactions is relatively low; in addition to the uncertainty introduced by the small number of animals used, it should be noted that all of the non-reacting animals in this series were sick during the course of the experiment. It appears that

TABLE VII

Influence of Quantity of Serum Injected upon Reactions in Rabbits' Ears

Serum injected pro kilo	3 cc.	5 cc.	7-8 cc.	10 cc.
Total.....	8	31	51	21
No. positive.....	0	22	36	13
No. negative.....	8	9	15	8

when the animals are not in good health during the experiment the reactions tend either to be questionable or negative. Many of the animals which are noted as showing questionable erythematous reactions were sick, and as already stated such reactions have been listed as negative ones. It does not appear that the introduction of the serum directly into the vascular system tends to increase the occurrence of the reactions, when compared with other routes of introduction.

In Table VII is shown the number of rabbits developing a positive reaction subsequent to the injection of varying quantities of horse serum. As stated previously the quantity injected was based upon the weight of the animal and we used varying amounts pro kilo. The animals injected with 3 cc. pro kilo showed no positive reactions. There is certainly no evidence that injections of increasing amounts of serum tend to increase the occurrence of the reaction; possibly

rather the reverse appears from the figures but whether this would hold in a larger series of animals is questionable. Certainly if a minimal adequate amount (accepting 5 cc. pro kilo as such) is injected the occurrence of reactions is practically maximal.

The first appearance of the reactions was usually manifested by the erythematous reactions, at times however edema appeared alone, or frequently the erythema and edema were noticed simultaneously. Therefore, in Table VIII in which we have shown the day after the single injection of serum on which positive reactions first appeared, in most cases this represents the appearance of erythema, often of

TABLE VIII
Number of Rabbits Showing Reactions on Various Days after Injection of Serum

Days.....	1	2	3	4	5	6	7	8	9	10	11
No. of rabbits.....	0	0	3	5	27	27	8	1	0	0	0

TABLE IX
Duration of the Reaction on the Rabbits' Ears

Days.....	1	2	3	4	5
Erythema.....	27	27	12	3	1
Edema.....	30	25	6	3	0
Erythema and/or edema.....	20	32	13	4	1

erythema and edema combined and only occasionally the appearance of edema alone.

It will be noted from Table VIII that very few reactions occurred before the 5th day and also relatively few after the 6th day. The vast majority of the reactions were first noted on either the 5th or 6th days.

These reactions varied markedly in duration, in some cases lasting for as long as 4 or even 5 days. It must be made clear that both erythema and edema did not always last equally long in the same animal. In Table IX the duration of the reaction is given and it is evident here that the two types of reaction were not of equal duration.

During the periods that the reactions were present there might occur variations in the degree of erythema, or of the edema, or one might disappear and the other persist. It can then be stated that the reactions showed at times periods of exacerbation and remission without at any time during their duration showing complete disappearance. We have never noticed what might definitely be considered a recurrence subsequent to disappearance of the reaction. In a few animals (20 in all) suspicious reactions of erythema and edema were noted on the 9th to 16th day after injection of the horse serum. However, these reactions cannot be considered as typical on the basis of the criteria which have been outlined above; the location, extent and general appearance left doubt as to their being typical and for these reasons we have not considered them as recurrent reactions. It may be that the criteria are too strictly drawn, but until considerable additional evidence is available it seems preferable to exclude these reactions noted at or after the 9th day subsequent to the injection, from the classification of reactions due to the injection of the serum.

It was stated above that a series of rabbits were injected with various antisera.³ In this series of 33 rabbits typical reactions were noted in eleven animals, or in 33 per cent. A larger proportion of these reactions occurred in a group injected with the concentrated antistreptococcus serum in contrast with a group injected with the antidiphtheritic serum. Since, however, in this latter group there were many rabbits with colored ears (brown) and since there is the definite suggestion that such animals do not react as well as do rabbits with white ears, we hesitate to lay stress upon the percentage difference noted when comparing the group injected with normal horse serum with the group injected with the two antisera. We are planning a further study of reactions subsequent to the injection of various antisera.

The important fact is, however, that the reactions occur subsequent to a single injection of various antisera as well as subsequent to the single injection of normal horse serum.

³ We wish to express our appreciation of the kindness of Sharp and Dohme in supplying us with diphtheria antitoxin as well as normal horse serum and of the kindness of Eli Lilly and Company in supplying us with concentrated antistreptococcus serum and normal horse serum.

DISCUSSION

The reactions described above have repeatedly been referred to as reactions of serum sickness in rabbits. Naturally the justification for assuming that these reactions are analogous to those which characterize serum sickness in man must be made clear. One may set down briefly the following characteristics of the disease in man. (1) Serum sickness appears subsequent to even a first injection of the foreign protein into man. (2) Serum sickness appears after an incubation period of from 4 to 12 days or possibly even a longer period. (3) The cardinal symptoms of serum sickness in man are urticarial reactions, erythematous reactions which vary considerably in intensity and area of involvement. (4) Associated with these cardinal symptoms but occurring less constantly there occur a rise of body temperature, arthralgia associated with swelling of the joints, swelling of lymph nodes, edema involving often the face and usually the looser tissue and certain other less obvious reactions. (5) The reactions may show variations from day to day, even to a disappearance and subsequent reappearance. (6) After the reactions have disappeared the individual is apparently restored to a normal state. (7) Serum sickness may appear in the immediate or accelerated form (within 8 hours or within 24 to 48 hours respectively) when the protein is injected for a second time into man, provided there has been a lapse of time between the first and second injections.

In the experiments reported here the reaction of the rabbits was subsequent to a first injection of a single large dose of horse serum. In a number of experiments serum was taken from the rabbits before the injections of horse serum were given and tested for the presence of precipitins; in no animals were precipitins demonstrable. It seems reasonably certain that we were in the case of the reacting rabbits not dealing with reactions of preexistent hypersensitiveness but with a reaction essentially analogous to that appearing in man after a first injection of serum.

The symptoms of serum sickness in man appear after an incubation period of several days. According to Longcope (3) the majority of the reactions occur on the 6th to 10th day after injection. Coca (4), states that 24 to 48 per cent of reactions in man appear less than 8

days after injection and only 14 per cent later than 12 days after. Sturtevant (5) in his study of 500 patients treated with diphtheria antitoxin noted serum sickness as occurring as early as the 1st day after treatment; the greater number of cases appeared from the 5th to the 9th day. Weaver (6) in a large series of patients given diphtheria antitoxin also reported reactions in the first 5 days but the majority of reactions appeared on the 6th to 10th days. It is evident that in the rabbits injected with horse serum a very large number showed reactions on the 5th and 6th days and only a few on either the 3rd, 4th, 7th or 8th days. The interval between injection and appearance of the reaction is therefore not exactly alike in man and rabbit, but there is a marked similarity in the incubation period in the two species.

The reactions appearing on the rabbits' ears subsequent to the injection of horse serum are characterized by erythema and edema. It is true that in man urticarial rashes are probably the most constant phenomena. We have never noticed in rabbits any reactions which might be considered as responses to the itching associated with urticaria; that is, excessive scratching of any parts of the body. However, the erythema on the rabbits' ears was as has been stated previously often of a morbilliform or scarlatinal type and certainly may be considered as similar to the erythema noted in man. Whether the edema noted in the rabbits' ears should be considered as analogous to the urticarial edema in man or to the edema seen so often about the face is not determinable, but it might in view of the frequency with which it occurs seem more like the former. It is true that in rabbits the edematous areas were not sharply outlined and were not pale and therefore differ distinctly from urticarial wheals. It is probable, however, that the edema of the rabbits' ears may be considered as similar to one or another of the reactions occurring in man.

Symptoms suggesting arthralgia, or evidence of swollen joints, were not noted in any of the animals, although careful observations were made in order to note any such reactions. It was not possible to be assured that in any of the injected or reacting rabbits the lymph nodes were enlarged; the difficulty of palpating the axillary or inguinal lymph nodes made it impossible to reach a conclusion in this regard. However, in one rabbit which before injection showed a chain of enlarged lymph nodes running from the axilla to the groin, subsequent

to injection and at a time when the reaction appeared on the ears these lymph nodes were distinctly enlarged, were softer than they had been previous to the injection, giving distinctly the feeling that inflammatory reactions were occurring about them. After the disappearance of the reactions on the ears the nodes again seemed to diminish in size and become hard as they had been previously. This isolated observation does not justify the conclusion that the lymph nodes are affected in rabbits as in man following the injection of serum. Possibly however it may seem to raise the question as to whether the enlargement of lymph nodes associated with serum sickness in man occurs in normal nodes or in abnormal ones; we should keep in mind the fact that serum sickness is usually produced in individuals suffering from an infection or an intoxication.

The temperature reactions of the injected rabbits have been studied and will be reported in a later communication.

It is apparent from the discussion above that the reactions occurring in rabbits are at least in part very similar to the cardinal reactions in man. The variations from day to day of the extent and intensity of the reactions of serum sickness as noted in man are distinctly more marked than the variations noted in rabbits. However, in some of the rabbits, on second observation some 8 to 10 hours after first being noted, the intense erythema would become distinctly less marked, only to return to its original intensity about 24 hours after it was first noted. In a similar fashion, but less frequently, the edema showed variations in degree from one observation to another, waxing and waning to an evident extent. After the reactions in the rabbits' ears had disappeared there was no evident residual injury, just as in man.

In a small number of rabbits which received a second injection of horse serum about 3 to 7 weeks after the first one, reactions in all respects identical to those which we have described above appeared either within 8 hours after injection or within 24 to 72 hours. These reactions are certainly analogous to the immediate and accelerated reactions of serum sickness in man.

In view of the similarities which have been pointed out above between serum sickness in man and the reactions noted on the rabbits' ears it appears certain that these phenomena can be considered as manifestations of serum sickness in rabbits.

CONCLUSIONS

1. The injection of a single large dose of normal horse serum into rabbits results in the appearance 3 to 8 days later of erythematous and edematous reactions on the ears in 68.9 per cent of the animals.
2. The injections may be given by any of several routes and reactions appear when the site of injection is definitely distant from the ears.
3. Injections of various antisera into rabbits cause the appearance of similar reactions.
4. These reactions can be considered as manifestations of serum sickness in rabbits.

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EXPLANATION OF PLATE 54

FIG. 1, *a* and *b*. *a*, left ear of normal rabbit. *b*, left ear of injected rabbit showing erythema of serum sickness; scarlatinal in basal third; mottled to morbiliform in two middle quarters.



a



b

FIG. 1

PLATE 54. FIG. 1.

DURATION OF DEMONSTRABLE ANTIBODIES IN THE SERUM OF RABBITS IMMUNIZED WITH HEAT-KILLED TYPE I PNEUMOCOCCI

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In a previous paper¹ have been reported the results obtained, as regards the development of agglutinating and protective antibodies in the blood, when rabbits are immunized by intravenous, intra-peritoneal, intramuscular or subcutaneous injections of heat-killed Type I pneumococci. The duration of time that these antibodies persist in the blood was not discussed, however. In the present study rabbits immunized by these various methods have been studied over long periods of time, until the antibodies have disappeared from the blood or until the animals have died from extraneous causes.

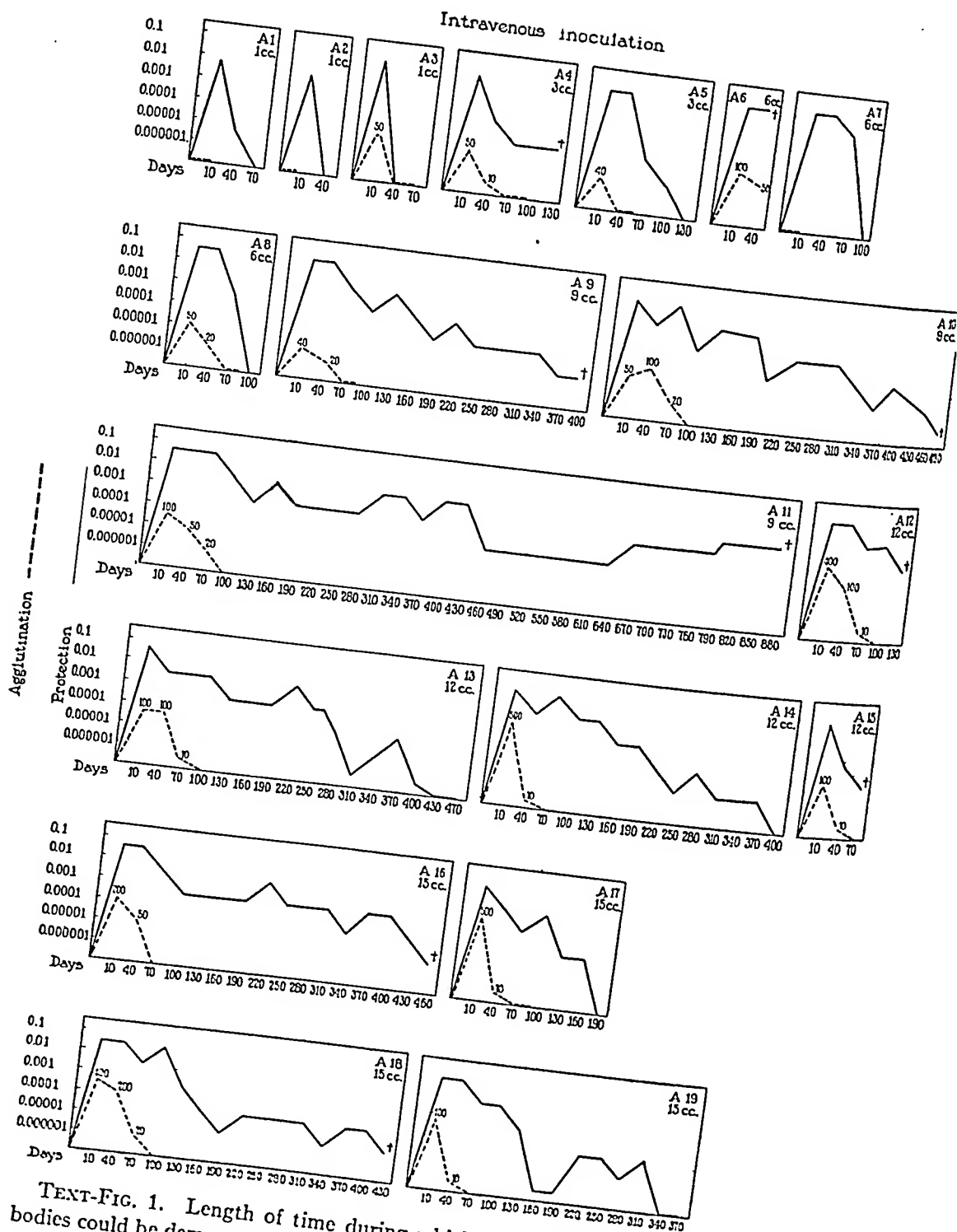
The methods employed were identical with those used in the previous study. The animals were bled 10 days after the last injection and thereafter every 30 days. The immunity response and the persistence of immune bodies in the blood in the rabbits of the several groups are shown in Text-figs. 1 to 4.

Rabbits Injected Intravenously

In Text-fig. 1 is shown the course of development and disappearance of antibodies in the serum of nineteen rabbits which received, during the course of treatment, from 1 to 15 cc. of the suspension of heat-killed organisms. Nine of the rabbits died from various causes before the completion of the observations.

It is evident that agglutinins were demonstrable during only relatively short periods following the immunization. In Rabbit A-14, which received 12 cc. of the bacterial suspension, the agglutination titre of the serum after 10 days was 1:500 but after 30 days the titre had decreased to 1:10, and on the 70th day no agglutinating power

¹ Stillman, E. G., *J. Exp. Med.*, 1930, 51, 721.



TEXT-FIG. 1. Length of time during which agglutinins and protective antibodies could be demonstrated in sera of rabbits which had been immunized intravenously with heat-killed Type I pneumococci.

whatever could be detected. In no instance could type-specific agglutinins be demonstrated longer than 100 days.

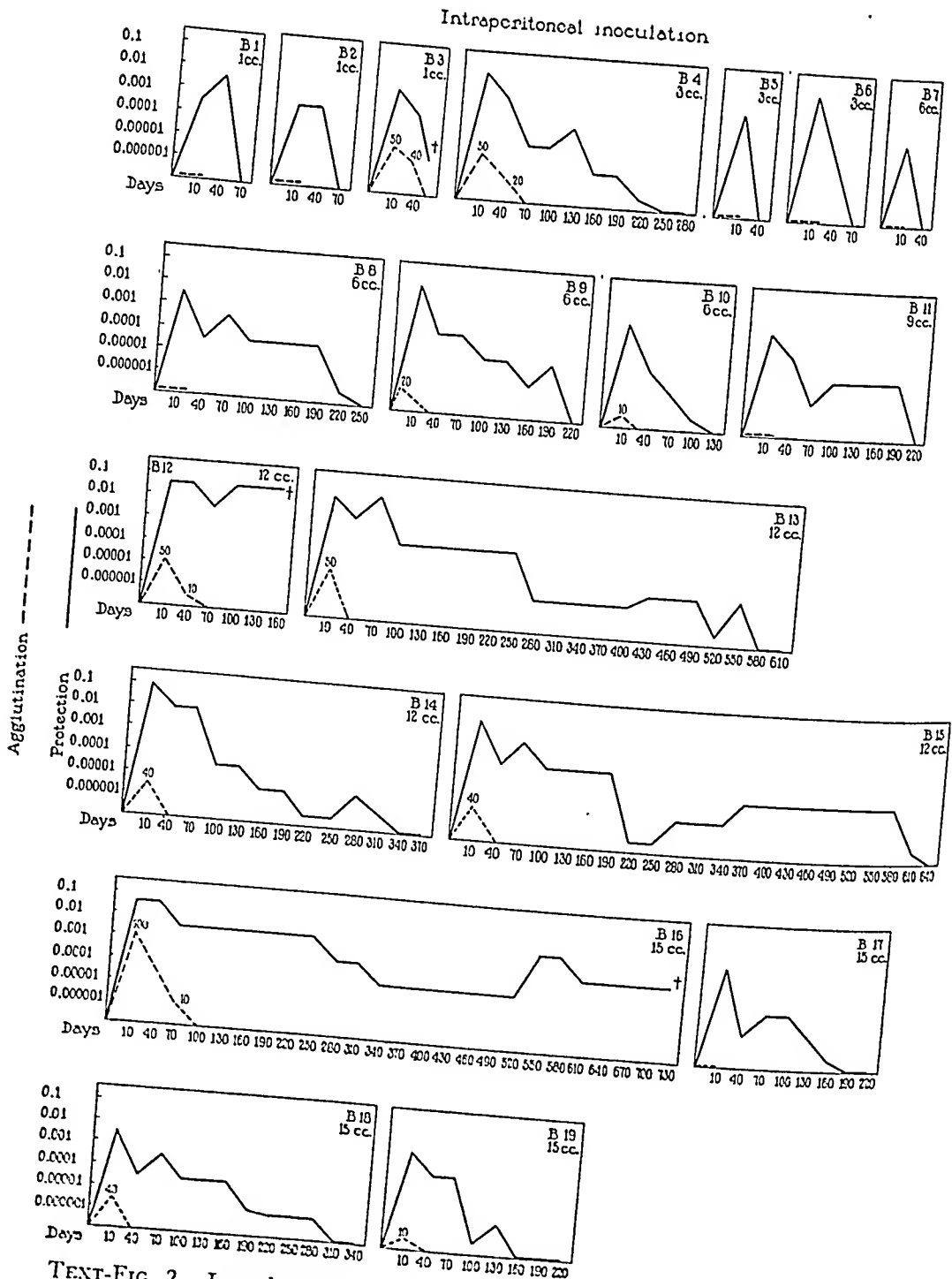
10 days after the course of immunization was ended the sera of all of the animals of this group showed well marked protective power. It is remarkable that the sera of the rabbits which received a total of 1 cc. of the bacterial suspension showed as high a titre of protective antibodies as did the sera of those which received as much as 15 cc. In the animals which received the larger amounts, however, the protective substances persisted longer than they did in the animals which received smaller amounts. In Rabbit A-11 which received 9 cc. of the suspension, the serum showed high protective power 880 days after the completion of the immunization.

Rabbits Immunized Intraperitoneally

The results in the rabbits immunized by intraperitoneal injections are shown in Text-fig. 2. All but three of these animals lived until demonstrable antibodies had disappeared from the blood. In only ten of the nineteen animals of this series were agglutinins demonstrable and they never reached as high a titre, nor did they persist so long, as in the animals injected intravenously. Protective antibodies were present, however, in the sera of all the animals, and, in the animals which received more than 6 cc. of the suspension, the protective action of the serum was demonstrable for long periods. Sera obtained from two of the rabbits 550 days after the course of immunization was completed still protected mice against 0.0001 cc. of culture, and in the case of one rabbit, serum obtained 730 days after immunization still exhibited protective action.

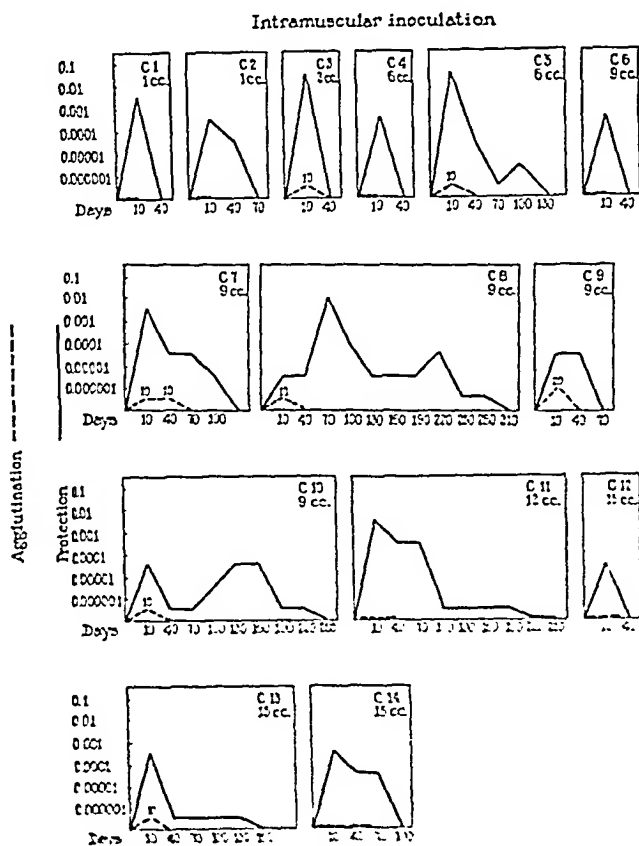
Rabbits Immunized Intramuscularly

Text-fig. 3 shows the number of days during which agglutinins and protective antibodies were detectable in the sera of fourteen rabbits immunized by intramuscular injections. All these animals survived until antibodies could no longer be demonstrated in their blood. The agglutinin titre of the sera from the rabbits of this series was never high and the presence of agglutinins could be demonstrated for only short periods. In fact, in the serum of only one of the rabbits could agglutinins be demonstrated as long as 40 days. The results as re-



TEXT-FIG. 2. Length of time during which agglutinins and protective antibodies could be demonstrated in sera of rabbits which had been immunized intraperitoneally with heat-killed Type I pneumococci.

gards the development of protective power were also irregular. Although, in a considerable number of the animals serum obtained 10 days after immunization was highly protective, this property was quickly lost. In only two of the rabbits could protective substances be demonstrated 160 days after inoculation.



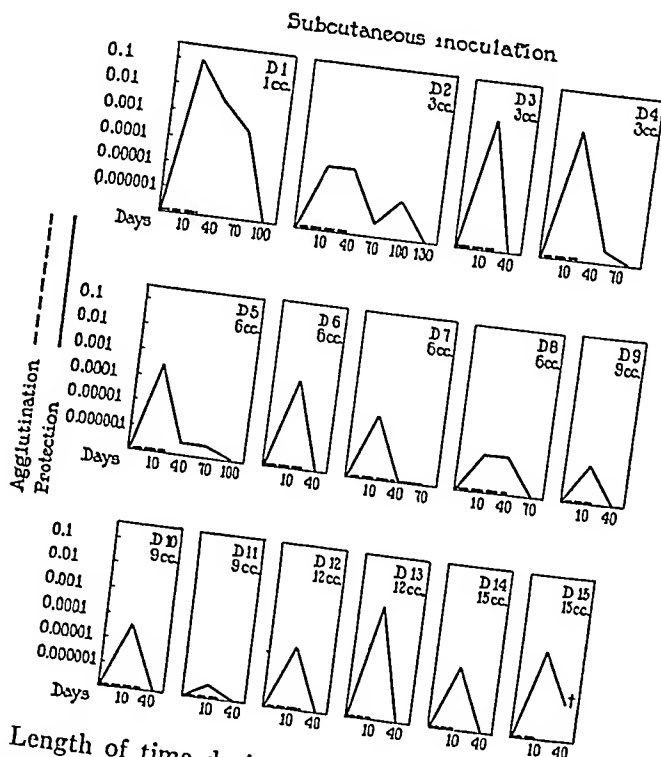
TEXT-FIG. 3. Length of time during which agglutinins and protective antibodies could be demonstrated in sera of rabbits which had been immunized intramuscularly with heat-killed Type I pneumococci.

Rabbits Immunized Subcutaneously

The results in fifteen rabbits which received subcutaneous injections are shown in Text-fig. 4. In the sera of none of these rabbits

DURATION OF ANTIBODIES IN SERUM

were agglutinins demonstrable at any time. Protective antibodies also were present only in low concentrations and they rapidly disappeared. In this series the animals which received the smaller doses not only showed higher concentrations of protective antibodies in the serum than did the animals which received the larger amounts but the antibodies persisted longer.



TEXT-FIG. 4. Length of time during which agglutinins and protective antibodies could be demonstrated in sera of rabbits which had been immunized subcutaneously with heat-killed Type I pneumococci.

DISCUSSION

These experiments show that in rabbits immunized by the injection of heat-killed organisms type-specific agglutinins are demonstrable in the serum only for short periods of time, though the actual length of time that these antibodies persist depends, to some extent, upon the route of immunization employed. In general, the agglutinin titre is highest and the agglutinins persist longest in the animals immunized by intravenous injections; the agglutinins are present in less high titre

and they persist for shorter periods in the animals immunized by the intraperitoneal route; they are still less regularly demonstrable and they persist for still shorter periods in the animals immunized by intramuscular injections; while in the animals immunized subcutaneously agglutinins are never demonstrable by the methods we have employed.

It appears that the injection by any route of a suspension of killed pneumococci will stimulate the production of protective antibodies and that when only 1 cc. of the suspension is injected the serum will be of as high a titre as when 15 cc. is injected. The length of time during which protective antibodies persist in the serum, however, varies with the route of injection and the amount of the inoculum. They persist longer in the animals receiving intravenous or intraperitoneal injections than they do in the animals injected intramuscularly or subcutaneously and in general they persist longer in the animals receiving larger amounts of the suspensions than in those injected with smaller amounts.

CONCLUSIONS

In rabbits immunized by the injection of suspensions of heat-killed pneumococci the results obtained as regards not only the development of agglutinating and mouse protective antibodies, but also the persistence of these bodies in the blood depend to a considerable extent upon the route of immunization and the size of the inocula. Agglutinins may appear in the sera of all the rabbits except those inoculated subcutaneously, but in most instances they disappear within a short time.

Protective antibodies appear in the sera of all rabbits, no matter which route of injection be employed and they persist much longer than do the agglutinins. They persist longest when the injections are made intravenously or intraperitoneally and are of briefest duration when the injections are made subcutaneously.

LOCALIZATION OF PNEUMOCOCCI IN THE LUNGS OF PARTIALLY IMMUNIZED MICE FOLLOWING INHALATION OF PNEUMOCOCCI

By ERNEST G. STILLMAN, M.D., AND ARNOLD BRANCH, M.D.
(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, June 3, 1931)

In preceding papers¹ it has been shown that when mice are intoxicated with alcohol and then sprayed with a culture of virulent Type I or Type II pneumococci many of the animals die with a general blood infection, but there rarely occurs any localization of the infection in the lungs. On the other hand, when mice, which have previously been exposed to inhalations of pneumococci of either type, are alcoholized and sprayed, pulmonary localization of the infection with manifest lesions in the lungs often occurs. There are at least two possible explanations of this phenomenon. It is possible that as a result of the first inhalation the tissues of the lungs become sensitized, or altered, so that on the second exposure the infection becomes localized in this organ. The second possibility is that by the first inhalation there is induced a state of general immunity, even of mild grade, and, as is well known, infections in immune animals are likely to be localized at the site of injection. Previous experiments have shown that after repeated inhalations of living virulent Type I pneumococci mice become increasingly resistant to infection with these organisms.

Experiments were undertaken, therefore, to determine whether pulmonary lesions may occur following the inhalation of virulent pneumococci by alcoholized mice that have previously been specifically immunized by routes other than the pulmonary one. In these mice a state of pulmonary sensitization cannot be assumed to be present. Mice were given intraperitoneal injections of (1) normal horse serum (2) Type I or Type II antipneumococcus serum or (3) Type I or Type

¹ Stillman, E. G., *J. Exp. Med.*, 1924, 40, 553, 567.

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LOCALIZATION OF PNEUMOCOCCI IN LUNGS

II pneumococcus vaccine, and after 10 days they were alcoholized and exposed to a spray of homologous or heterologous pneumococci.

EXPERIMENTAL

Methods.—Normal horse serum or antipneumococcus horse serum diluted in normal salt solution was injected intraperitoneally into mice in amounts varying from 0.1 cc. to 0.0000000001 cc., the total quantity of fluid injected in each case being 0.5 cc. 3 hours later the animals were alcoholized and exposed to a spray of pneumococcus culture. Other mice were injected intraperitoneally with a suspension of heat-killed pneumococci in doses varying from 0.1 cc. to 0.0000000001 cc. The vaccine was so prepared that 1 cc. of the suspension was equal to 10 cc. of broth culture. 10 days later these mice were intoxicated with alcohol and exposed to a spray of a culture of virulent Type I pneumococci.

All the mice were alcoholized by the intraperitoneal injection of 1.5 cc. of a 10 per cent solution of alcohol in salt solution. 1 hour after the administration of the alcohol the animals were placed in the chamber and sprayed with 50 cc. of the broth culture of virulent *Pneumococcus* Type I. They were exposed in the spray box for 1 hour. In every instance in which fatal infection occurred, cultures were made from the heart's blood, and at least one lobe of the lung was fixed in Zenker's fluid for histological study.

Pulmonary Lesions in Infected Mice Which Had Received Normal Horse Serum

In order to determine if the injection of a foreign protein as such would so change the reactivity of mice that pneumococci would localize in the lungs, 201 mice were first injected intraperitoneally with normal horse serum, then alcoholized and exposed to infection by the spray method. Although twenty-five, or 12 per cent of these mice, died of pneumococcus septicemia, no localized lesions in the lungs were present.

Pulmonary Lesions in Passively Immunized Mice

215 mice were passively immunized by a single intraperitoneal injection of Type I antipneumococcus serum, various amounts being employed. They were then alcoholized and sprayed with an homologous culture of Type I pneumococcus. Of the mice so treated, forty-eight, or 22 per cent, died of pneumococcus septicemia, and twelve, or 25 per cent of those dying, showed definite, localized, pulmonary lesions.

In order to determine if pulmonary localization could be produced by the use of immune serum of a heterologous type, mice were similarly injected with Type II antipneumococcus serum, alcoholized and sprayed with a culture of Type I pneumococci. Of the 224 mice so treated, seventeen, or 7 per cent, died of pneumococcus septicemia, but in no instance was there any evidence of pulmonary localization. It appears, therefore, that the injection of heterologous immune serum does not afford sufficient immunity to cause a localization of the pneumococcus infection in the lungs.

Pulmonary Lesions in Actively Immunized Mice

Of 211 mice, previously injected intraperitoneally with varying amounts of a suspension of heat-killed Type I pneumococci and subsequently exposed to a spray of a living culture of pneumococci of the homologous type, thirty-two, or 15 per cent, died of pneumococcus septicemia, and pulmonary localization occurred in four, or 12 per cent of those dying.

Pulmonary Lesions in Mice Actively Immunized by the Injection of Suspensions of Heat-Killed Pneumococci of a Heterologous Type

In order to determine if localization would occur in mice actively immunized by the injection of a suspension of heat-killed pneumococci of a heterologous type, 215 mice were injected with a suspension of heat-killed Type II pneumococci, and 10 days later all the animals were alcoholized and sprayed with a culture of virulent Type I pneumococcus. 52, or 24 per cent of these mice, died of pneumococcus septicemia but in none was there any evidence of an attempt to localize the infection.

DISCUSSION

Table I shows in a condensed form the results obtained in these experiments. It is seen that considerable variation occurred in the frequency of death. At first sight it is difficult to see why only 12 per cent of the animals receiving normal horse serum died, while of those that received immune serum 24 per cent died, and only 7 per cent of those that received heterologous serum. It must be remembered, however, that in infecting animals by the inhalation method:

is impossible to control the dosage accurately. There must be great variation in the number of organisms that lodge in different parts of the respiratory tract and also in the number of bacteria that actually invade the tissues. The mice in each experiment were divided into groups, each group of fifteen to twenty mice being treated exactly alike, but the mice of the different groups received different amounts of normal, or immune serum, or heat-killed bacteria. When all these circumstances are taken into consideration it is not surprising that there should have resulted considerable irregularity in the results, so far as protection is concerned, though it is not believed that these results invalidate the conclusions regarding the localization of the infection in the lungs.

TABLE I

The occurrence of pulmonary lesions in mice alcoholized and sprayed with culture of Type I pneumococci after receiving injections of normal serum or homologous or heterologous immune horse serum, or after active immunization by injections of homologous or heterologous heat-killed pneumococcus cultures.

Treated with	No. of mice	No. died	Per cent	No. having pulmonary lesions	Per cent of these dying
Normal horse serum.....	201	25	12	0	0
Homologous immune horse serum.....	215	48	24	12	25
Heterologous immune horse serum.....	224	17	7	0	0
Homologous vaccine.....	211	32	15	4	12
Heterologous vaccine.....	215	52	24	0	0

The fact that 25 per cent of the animals which died after receiving homologous immune serum and 12 per cent of those receiving homologous vaccine exhibited localized pulmonary lesions, while none of those previously treated with normal horse serum, heterologous serum or killed bacteria showed pulmonary lesions can hardly be explained by chance alone. The experiments seem to show that slight grades of specific immunity are important in causing the organisms to localize in the lungs and to produce lesions there.

CONCLUSIONS

1. When mice are passively immunized by the intraperitoneal injection of antipneumococcus horse serum or actively by the injection

of heat-killed pneumococcus cultures, and are then alcoholized and sprayed with a culture of pneumococci of the same type as that of the bacteria employed in immunization, a considerable number die with localized lesions in the lungs.

2. If instead of injecting immune serum of the type corresponding to that of the bacteria employed in producing the infection, normal horse serum or immune serum of a heterologous type be injected, or if the animals be previously immunized by the injection of killed pneumococci of a heterologous type, none of the animals which die show any evidence of localization of the infection in the lung.

3. The occurrence of pulmonary lesions in alcoholized mice after spraying with a culture of pneumococci is the consequence of a general immunity of a very mild grade.

THE ESTABLISHMENT OF AN ALBINO RAT COLONY FREE FROM MIDDLE EAR DISEASE

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(Received for publication, July 11, 1931)

Earlier experimental studies on middle ear disease in the albino rat were hampered by the high natural incidence of the condition, particularly among adult animals.¹ It was apparent that a population free from nasal involvement was essential for interpreting precisely

CORRECTION

In Vol. 54, No. 5, November 1, 1931, page 715, first line of fourth column in Table II (under "Anticoli horse serum"), for H₆₀Bl₁₈₉, read H₆₀Bl₁₈₈.

of us (Gowen), which furnished the original data on the incidence of middle ear disease.¹ This colony comprises a number of lines which are preserved intact with no cross breeding.

Young breeders under 6 months of age chosen from several different lines were mated and placed in individual cages. The pregnant females were subsequently removed and isolated in a separate unit. The males meanwhile were killed and autopsied. The suckling rats were left with their mothers until old enough to wean. At this time the female parents were killed and examined. The young rats from a single pair of parents free from middle ear disease and pneumonia consti-

¹ Nelson, J. B., and Gowen, J. W., *J. Infect. Dis.*, 1930, 46, 53.

² Nelson, J. B., *J. Infect. Dis.*, 1930, 46, 64.

tuted the nucleus from which the selected colony was developed. They were born early in January, 1929. Brother and sister matings were subsequently made through seven generations. Several pairs of breeders from each generation were selected and segregated from the surplus stock of special rats. The breeders were all killed after their young were weaned and examined for middle ear disease and pneumonia.

From time to time small groups of rats were removed from the selected colony and killed. At autopsy, particular attention was paid to the condition of the upper respiratory tract, the middle ear, and the

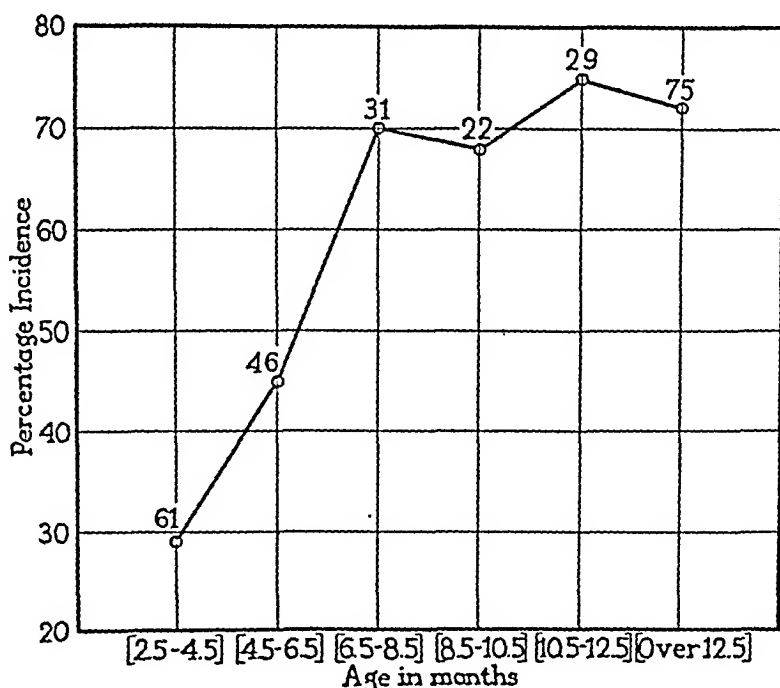


FIG. 1. The incidence of middle ear disease in stock rats of different ages. The number of rats in each age group is placed above the plotted morbidity rate.

lung. This series of autopsies now contains 120 individuals varying in age from 2 to 16 months and about equally divided as to sex. A few rats showed an increased mucus secretion in the nasopharynx or the nasal canals, but in general the upper respiratory tract was normal. The middle ear cavities throughout the entire series were normal in the gross. The tympanic membranes consistently gave the characteristic click upon puncture with the scissors point. Microscopic examination

of saline washings from the tympanic cavity, which was made in some instances, gave no indication of a cellular reaction. In no case was there an involvement of the tympanic bone, inner ear, or internal meatus. No rats were observed with the inclined position of the head or the rotary motion characteristic of the so called twister.

A similar postmortem examination of rats from the stock colony has been carried out since 1928. This series likewise included rats of all ages up to 2 years. Over half of the animals were contemporary with those of the selected population. This series now contains 264 rats, of which 152, or 57 per cent, showed an inflammatory involvement of one or both middle ear cavities.

TABLE I

The Incidence of Middle Ear Disease and Pneumonia in Adult and Young Rats from the Selected and Stock Colonies

	Adult rats					Young rats				
	No. of rats	Middle ear disease		Pneumonia		No. of rats	Middle ear disease		Pneumonia	
		No. of cases	Percentage incidence	No. of cases	Percentage incidence		No. of cases	Percentage incidence	No. of cases	Percentage incidence
Selected colony.....	50	0	0	26	52	50	0	0	0	0
Stock colony.....	50	36	72	39	78	50	15	30	0	0

It was previously shown that the incidence of middle ear disease in the albino rat increased significantly with age.¹ This is brought out in Fig. 1 which shows that the morbidity rate rises steadily to about 8 months and then remains nearly stationary at a high level.

Bearing in mind the effect of age on the incidence of middle ear disease, the rats removed from the special colony for autopsy were generally selected from two age groups, young rats 3 to 4½ months old and adult rats upwards of 1 year in age. The data on middle ear disease and pneumonia within these age limits in the selected and stock colonies are summarized in Table I. The series from the stock colony was composed of the last fifty animals in each age group examined at autopsy.

AN ALBINO RAT COLONY

No cases of middle ear disease were found in the selected rats of either age group. The adult rats from the stock colony showed a high incidence, approximately 70 per cent. In the young rats from the same colony it was significantly lower, 30 per cent. Approximately 50 per cent of the adult selected rats showed pneumonic lesions in one or more lobes of the lung. The incidence of pneumonia in the adult stock rats was appreciably higher, 78 per cent. No cases of pneumonia were found among the young rats of either colony. In the past a few cases were observed in rats of this age, 3 per cent in a group of 70.¹

It was of interest to know whether middle ear disease was present among the stock rats of the line from which the selected colony was developed. Inspection of the autopsy records showed that thirty-six rats from this line were examined. Sixteen, or 44 per cent, of these animals showed middle ear disease. The difference in the morbidity rates of this line and the stock group as a whole may be due in part to the chance inclusion of a greater number of young rats in the former. From these findings there is no indication that the rats of the selected line were more resistant to middle ear disease than the stock rats as a group.

The incidence of pneumonia in the adult selected rats was approximately a third less than that in the adult stock rats. The postmortem examinations suggested that in addition the disease was less advanced in the selected rats. The autopsy data were therefore analyzed to determine whether any significant difference in the extent or severity of the pneumonia could be brought out.

In order to compare the extent of the pneumonia in the adult rats of the two colonies, the number of affected lobes in individual cases was determined. The number of cases which showed involvement of two or more lobes was less among the selected rats but the difference was not significant. Thirteen, or 50 per cent, of the twenty-six adult selected rats affected with pneumonia showed involvement of two or more lobes, and twenty-three, or 58 per cent, of the thirty-eight adult stock rats. The two groups were then compared on the basis of the type of lesion most commonly encountered, with more significant results. Advanced lesions occurred approximately two and a half times as often among the adult stock rats. Twenty-five, or 63 per cent, of the selected adult rats displayed nodular lesions containing a

muroid or purulent exudate, and only seven, or 23 per cent, of the affected rats from the selected colony were similarly affected.

Although the rats of the selected colony showed no evidence of middle ear disease, it was possible that carriers of the associated bacteria might be present. The obscure etiology of the condition made it impossible to employ direct methods for their detection. The institution of a vitamin-deficient diet was resorted to with the possibility that its debilitating effect on the rat might predispose to the development of middle ear disease in the presence of the infecting bacteria.

Twenty young rats from the selected colony, approximately 2 months of age, were placed on a diet deficient in vitamin D. The Steenbock rachitic ration containing 76 parts of yellow corn, 20 of wheat gluten, 3 of calcium carbonate, and 1 of sodium chloride was employed. The rats were housed in wire cages, two to four individuals to a cage, and were isolated in a unit separate from the main se-

TABLE II

The Incidence of Middle Ear Disease in Rachitic Rats from the Selected and Stock Colonies and in Stock Rats on a Balanced Diet

	No. of rats	No. of cases	Percentage incidence
Rachitic selected rats.....	20	0	0
Rachitic stock rats.....	45	22	48
Normal stock rats.....	50	15	30

lected colony. The rachitic diet was maintained throughout their lives. Sixteen died between 4 and 6 months of age, and the other four were killed after 6½ months. All showed characteristic deformation of the hind legs and ribs. Post-mortem examination was made in every case.

The experimental findings are summarized in Table II. For comparison, the incidence of middle ear disease in two other groups of rats is also shown. One group is composed of young stock rats from a number of different lines which were maintained on a similar rachitic diet. It represents one age group of a much larger series; in particular, rats which died between 3 and 4½ months of age. The other, taken from Table I, is composed of rats of the same age maintained on a balanced diet. It may be noted that the rachitic stock rats were kept in separate cages in the same room with the main colony. After the

institution of the special diet they never came in direct contact with other rats of the colony.

No cases of middle ear disease occurred in the selected rats maintained on the rachitic diet. The rachitic stock rats, on the other hand, showed a high rate of middle ear disease, appreciably higher than the expected rate in stock rats of the same age group on a normal diet. Exposure of the rachitic rats to the infecting agents after they were placed on the special diet was solely by indirect contact, whereas the normally fed stock rats were directly exposed, or potentially so, through contact with their litter mates. The increased rate of middle ear disease in the former group suggests that a rachitic diet may predispose to the development of middle ear disease in a certain percentage of young rats which under normal conditions would not be affected.

Because of the negative evidence afforded by the above observations, one additional experiment was undertaken in the attempt to demonstrate the presence of carriers in the selected population. A prolonged state of overcrowding was chosen as an environmental factor operating against the defensive forces of the host and in favor of the development of such parasitic bacteria as might be present. Ten weaned, selected rats from two different litters were placed in a single cage (15 x 10 x 10 inches) and kept together for nearly a year. The animals were subsequently killed and autopsied. Examination of the middle ear cavities failed in every instance to show any indication of an inflammatory reaction.

DISCUSSION

Because of the high incidence of middle ear disease among the adult rats of the stock colony, it can be assumed that the total mass or dosage of associated bacteria, disseminated throughout the population, is large. The perpetuation of the condition from generation to generation is assured by the constant influx of younger susceptible rats.

The disappearance of middle ear disease from the selected colony was effected either by a prompt reduction of the associated bacteria to a non-infective level or by their complete elimination. Although it is possible that by chance a resistant line of rats was selected, the fact that rats from the same line as residents of the stock colony were affected with middle ear disease directly opposes such a view.

It is possible that the bacteria associated with middle ear disease may persist within the selected population, carried in the upper respiratory tract or elsewhere by a few rats. The subjection of selected rats to abnormal environmental conditions favoring the development and spread of infective bacteria failed, however, to bring out any cases of middle ear disease. Whether the present state of the colony with respect to the condition will be maintained in the future cannot be predicted. Unless the infecting agents are reintroduced in considerable numbers from the outside it may be supposed that the incidence of middle ear disease will be held at least to a low level.

The procedure followed in establishing the selected colony failed to eliminate pneumonia. The morbidity rate was lower than the normal rate for stock rats but was still high; approximately 50 per cent of the adults showed definite pneumonic areas in one or more lobes of the lung. The frequent development of pulmonary lesions without middle ear involvement strongly suggests that the primary inciting agents of the two infections are not identical. The group of selected rats maintained for a long period under conditions of overcrowding affords evidence against the unity of the infecting bacteria. 60 per cent of these rats showed pneumonia without a single case of middle ear disease. If the two conditions were caused by the same bacteria it would be necessary to assume either the rapid development of an acquired immunity following involvement of the lung, or the existence of a natural organ resistance in the selected rats. Neither assumption is in keeping with known facts. In a similar group of stock rats a nearly equal incidence of the two conditions would be expected. As previously noted, the strain of selected rats which remained in the stock colony was affected with middle ear disease.

Although the incidence of pneumonia was not markedly decreased in the selected rats, there was evidence that the pulmonary reaction was less advanced than that in the stock rats. It is suggested that the development of terminal lesions from the primary focus is accelerated or in part determined by the development of secondary invaders. Infection of the middle ear might well establish a locus for the spread of such bacteria. The removal of this locus from the selected rats may account for the retarded pneumonic reaction. In this connection it is of interest that *B. actinoides* variety *maris*, which was recovered at

times from the affected lungs of stock rats, was never isolated from the selected animals.

Each generation of young breeding rats which was removed from the main selected colony and kept under isolation was free from pneumonia. In the maintenance of rat colonies there is reason to believe that limiting the age of the breeding stock to approximately 6 months and removing old rats from the surplus stock would reduce the incidence of pneumonia to a low level.

SUMMARY

A special colony of albino rats was built up by selection and isolation from a population in which middle ear disease was highly prevalent. No cases of aural infection occurred in the selected group, whereas its precursor showed a crude incidence of 57 per cent. The subjection of selected rats to a rachitic diet and to overcrowding did not predispose to the development of middle ear disease.

The incidence of pneumonia was not similarly affected; thus, 52 per cent of the adult selected rats showed pulmonary lesions, and 78 per cent of the adult stock rats. There was, however, a significant reduction in the number of cases which showed advanced pulmonary lesions.

Certain theoretical considerations of middle ear disease and of pneumonia are discussed.

SPECIFIC CUTANEOUS REACTIONS AND CIRCULATING ANTIBODIES IN THE COURSE OF LOBAR PNEUMONIA*

I. CASES RECEIVING NO SERUM THERAPY

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(Received for publication, July 6, 1931)

In a recent paper Tillett and Francis (1) reported the finding of definite and characteristic cutaneous reactions to certain purified chemical fractions of the pneumococcus in patients recovering from lobar pneumonia. In the cases studied, they showed that the type-specific polysaccharide (the soluble specific substance, or "S.S.S.") injected intracutaneously at the time of crisis or later produces a "wheal and erythema" type of response which is specific for the type of pneumococcus causing the disease and that fatal cases fail to give such responses. The acetic acid precipitable fraction of the pneumococcus, the so called nucleoprotein, when injected at about the time of crisis or later elicits a delayed tuberculin type of reaction in all cases of pneumonia without respect to the type of pneumococcus causing the disease. They showed that these cutaneous reactions are associated with the presence of agglutinins and precipitins in the serum of the patient.

These findings suggested that the type-specific skin reactions to the pneumococcus polysaccharides might be useful in prognosis. Furthermore, Tillett and Francis suggested that "persistence of infection even though specific antibodies are present, may inhibit the skin response," intimating that such reactions might be used as a simple test for complete recovery from the specific infection.

This paper is concerned with: (1) The cutaneous responses to

* This study was aided in part by grants from the William W. Wellington Fund of the Harvard Medical School and from the Influenza Commission of the Metropolitan Life Insurance Company.

repeated skin tests with the specific carbohydrates of Types I, II and III pneumococci and circulating specific antibodies at various intervals in 41 cases of lobar pneumonia that received no specific therapy. (2) Similar studies on a single occasion early or late in convalescence in 44 comparable cases and in 24 individuals without recent pneumonia.

Material and Methods

Repeated tests were done in 41 patients admitted to the medical wards of the Boston City Hospital during the winter and spring of 1930. These patients all had typical lobar pneumonia, clinically and radiographically. A pneumococcus of either Type I, Type II or Type III was obtained in each case from the sputum or the blood culture, or both. No specific antipneumococcic agents were used in the treatment of these cases. There were 30 recovered and 11 fatal cases. 15 of the the former had Type I, 7 had Type II and 8 had Type III infection; of the 11 fatal cases, 5 were caused by Type I, 4 by Type II and 2 by Type III pneumococci. A positive blood culture was obtained in only 2 of the recovered cases (J. S., Type I, and J. K., Type II (Fig. 1); whereas, 9 of the fatal cases had a pneumococemia. The presence of complications will be mentioned later. The patients ranged in age from 13 to 58 years. Only 5 of the patients were females.

Skin tests were done on a single occasion 4 to 23 days after crisis in 35 cases of lobar pneumonia that received no specific antipneumococcic therapy, but were, in all respects, similar to the cases mentioned above. The sera of many of these cases were tested for antibodies during the acute disease and at the time of crisis, as well as at the time that the skin tests were performed. 9 other cases were studied on one occasion 5 to 14 months after recovery from pneumonia not specifically treated. 24 hospital patients with no recent history of pneumonia were studied. These included cases of peptic ulcer, chronic arthritis and blood and cardiac diseases. The ages of the latter group ranged from 13 to 77 years.

Skin Tests.—A 1:200 solution of each of the Types I, II and III purified specific carbohydrates was obtained from the Hospital of The Rockefeller Institute for Medical Research, through the courtesy of Dr. W. S. Tillett. 1:10,000 dilutions were prepared every few days from the stock solutions by diluting with physiological saline freshly made with redistilled water and then heating for 10 minutes at 100°C. to insure sterility. 0.1 cc. of each of these 3 solutions (containing 0.01 mg. of the carbohydrate) and of a control of the saline used in making the dilutions were injected intradermally on the flexor surface of the forearm. All 4 injections were repeated at various intervals during the acute disease and during convalescence. In a few instances where positive tests were obtained, higher dilutions up to 1:30,000,000 were used in order to determine the smallest amount with which a positive test could be obtained.

Antibody Determinations.—Serum was obtained from venous blood, drawn

shortly before, occasionally after, almost every test. The titer of agglutinins and of mouse protective antibodies for Types I, II and III pneumococci was determined in most instances. Agglutination tests were done by the method employed by Tillett and Francis (1). Protective antibodies were determined by the usual mouse method. Precipitin tests were done with the specific carbohydrates and with culture filtrates, but the results were irregular and are not reported.

Varieties of Cutaneous Reactions to the Specific Polysaccharides.—The intracutaneous injection of the type-specific carbohydrates usually produces no greater response than does the injection of an equal amount of physiological saline. The response occurring in reacting individuals recovering from lobar pneumonia has been described and illustrated by Tillett and Francis (1). Fairly wide variations in the appearance of the reaction were observed, in the course of this study, in different patients and at different times in the same patient. These variations were probably only differences in intensity, since mild reactions could be produced in patients who showed strong reactions to standard dosages by the injection of 0.0001 mg. The milder reactions consisted of a faint pink wheal, 6 to 10 mm. in diameter, which shaded into an area of faint erythema, 12 to 20 mm. in diameter. This type of reaction usually reached its maximum within 30 minutes and faded entirely before the end of 45 to 60 minutes. The intense reaction consisted of an almost white, edematous, sharply demarcated wheal irregular in outline giving the appearance of "pseudopods." 1 or 2 drops of serum occasionally oozed from the puncture wound of this type of case. This type of wheal was usually surrounded by an intense erythema, 4 to 6 cm. in diameter, the periphery of which faded into an area of faint mottling. Such striking reactions developed in 10 to 20 minutes and remained at their maximum intensity for 30 to 60 minutes, after which the wheal gradually blended with the surrounding erythema to form a fairly soft, usually non-tender, uniform swelling lasting 6 to 48 hours. The skin over the central portion of the swelling usually remained reddened. In rare instances ecchymosis appeared in the edematous area. Occasionally, although no reactions occurred on the day of the injection, erythema was observed 24 to 72 hours later at the site of injection of the carbohydrate which corresponded to the type of the infecting pneumococcus. Another test, performed upon noticing such a delayed reaction, elicited a typical immediate reaction with the same material.

Reactions were called doubtful when they were greater than that produced by the saline control but showed a wheal less than 0.8 cm. and a surrounding erythema less than 1.5 cm. in diameter.

Occurrence of Cutaneous Reactions to the Specific Polysaccharides

The results of all of the skin tests in the 30 recovered cases are charted in Fig. 1. Positive cutaneous reactions to the homologous type polysaccharides (derived from the same type as the pneumococcus causing the disease) were obtained in 17 of the 30 recovered cases. In

addition, 10 of the 17 patients who gave homologous positive tests also showed positive reactions to heterologous polysaccharides. In 2 other cases heterologous positive tests were observed but the homologous carbohydrates gave negative reactions. All the heterologous positive responses in cases due to Types I and III pneumococci were elicited with the Type II S.S.S. whereas the heterologous reactions in the Type

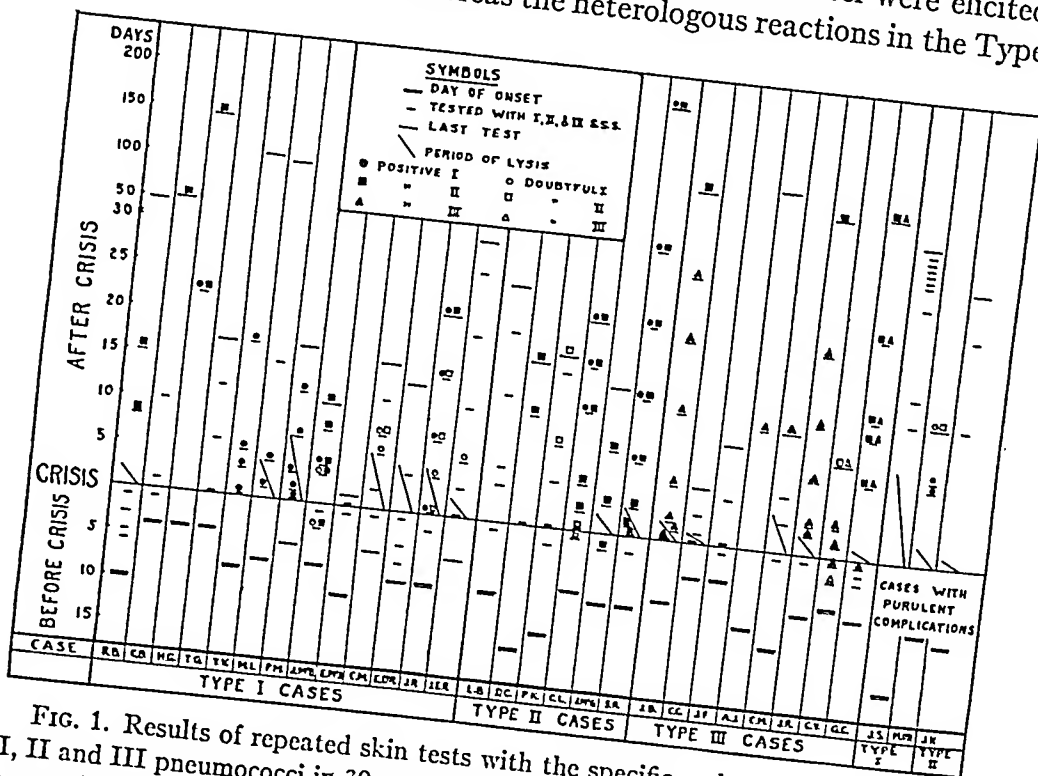


FIG. 1. Results of repeated skin tests with the specific carbohydrates of Types I, II and III pneumococci in 30 recovered cases of lobar pneumonia that received no specific therapy.

II cases occurred with the Type I polysaccharide. The type distribution of the cases and of the skin responses is summarized in Table I. There were 3 cases among the 30 that recovered who had purulent pneumococcal complications at the time of the first test. One of these 3 patients, a case with Type I pneumococcus empyema, gave positive tests with the homologous S.S.S. In the other 2 cases, one having purulent Type I pneumococcus arthritis and the other having an empyema and purulent conjunctivitis from both of which Type II

pneumococci were obtained, the reactions to all the carbohydrates were negative. There were also 2 cases that had sterile pleural effusions during convalescence. One of these cases, a patient with Type I pneumonia, gave negative reactions to all tests, whereas the other, a Type III case, gave positive tests with both the Type III and the Type II S.S.S.

Of the 11 fatal cases, only one showed a positive homologous test. In this patient characteristic positive tests were elicited 36 hours and 12 hours before death. Cultures taken at these times showed, respectively, 500 and 700 colonies of Type II pneumococci per cubic centimeter of blood. Heterologous positive tests were not obtained in any of the fatal cases.

Occurrence of Protective Antibodies and Agglutinins

Protection of mice against the homologous type organism was demonstrated against 100 lethal doses or more in the sera of all of the 24 recovered cases in whom tests were done on more than one occasion. Homologous agglutinins were present after recovery in all but 2 of the 27 cases tested in dilutions of serum up to 1:64. The 2 patients who had no agglutinins also had negative skin tests with the homologous polysaccharides (C. B., Type I, and J. F., Type III). Protective antibodies for heterologous types of pneumococci were demonstrated in 19 of the 25 recovered cases in which such tests were carried out. In 3 additional cases, protection against 10 lethal doses or irregular protection was found. Heterologous agglutinins were present in the sera of 17 of the 28 patients tested after crisis. The findings in the recovered cases are summarized by types in Table I.

All 3 of the recovered cases having purulent complications and both patients with sterile pleural effusions had protection for the homologous and heterologous type pneumococci after the presence of the complication was established. Agglutinins were demonstrable in the sera of all of these patients for the homologous type organism and in one of the latter patients for an heterologous type.

Among the fatal cases, homologous agglutinins were demonstrated only once. This was on the day of death in a Type I patient who had been ill 22 days and had a persistent low grade pneumococcemia and

infected pleural fluid (reported by Lord and Persons (2)). Protective antibodies for the same type were present in this case at the same time and also on 2 other occasions during the previous week. No autopsy was obtained on this patient. Homologous protection against more

TABLE I
Summary of the Cutaneous Reactions, Agglutinins and Protective Antibodies in 30 Recovered Cases Repeatedly Tested

Infecting type	Type I tests		Type II tests		Type III tests	
	No. of cases tested	Cases with positive tests	No. of cases tested	Cases with positive tests	No. of cases tested	Cases with positive tests
Cutaneous reactions						
I	15	7	15	5*	15	0
II	7	2	7	4	7	0
III	8	0	8	0	8	6
Agglutinins						
I	14	13**	14	9	14	1
II	7	1	7	7	7	2
III	7	0	7	4	7	6
Protective antibodies						
I	14	14	14	113‡	10	33
II	6	3	6	6	4	2
III	5	31	5	5	4	4

* 2 of these failed to show homologous positive tests, all other cases having positive tests with an heterologous S.S.S. had positive homologous tests as well.

** 2 had agglutinins only in 1:2 dilution of serum.

‡ Raised numerals indicate cases having protection for 10 lethal doses or irregular survivals among the mice in the higher dilutions.

than 10 lethal doses was not found in any of the other fatal cases. Heterologous antibodies were never demonstrated.

Relation of the Cutaneous Reactions and of Antibodies to the Course of the Disease

Appearance of the Cutaneous Reaction to Specific Polysaccharides.—A sensitive homologous test was elicited in only 3 of 17 cases tested before

the day of crisis.¹ In 2 of these cases, the reaction was obtained on the day before crisis, and, in the 3rd, it was elicited 2 days before the crisis. By the day of crisis, 11 of 25 patients tested showed positive reactions to the homologous S.S.S. Whereas 12 of 25 patients tested by the end of the first week after crisis had already shown homologous positive tests, only 1 (J. McB., Type I) had a positive test with an heterologous S.S.S. within this period. This patient's heterologous positive reaction was observed when he was first tested 3 days before crisis. In general, the positive tests with the homologous polysaccharides were first demonstrable at about the time of crisis and, in some cases, while the temperature was still elevated and the patients appeared acutely ill. On the other hand, heterologous positive tests were first elicited 8 or more days later, and often quite late in convalescence. In particular, 3 patients first showed heterologous positive responses 2, 3 and 4 months after crisis at a time when the tests with the homologous carbohydrates were already negative in each instance.

Duration of the Skin Reactivity to Specific Polysaccharides.—The last positive test with the homologous polysaccharide was elicited less than 1 month after crisis in 15 of the 17 cases in which homologous reactions occurred (Fig. 1). Subsequent negative tests with the same types occurred in 9 of these 15 cases. Positive homologous tests were elicited in only 2 of the 8 patients tested after 3 months of convalescence. Reactions to heterologous polysaccharides, on the other hand, persisted in all but 1 of the 10 patients as long as observations continued; in 5 cases more than 3 months and in a 6th case 51 days after crisis.

The duration of the skin reactivity varied with the type of polysaccharide injected. Type II reactivity was more lasting than that elicited by the Type I and III carbohydrates. It will be seen that 6 of the 9 patients having positive reactions to the Type I polysaccharide gave negative responses subsequently and 2 of the remaining 3 were last tested only 22 days after crisis. Only 1 case (S.R., Type II) gave a positive reaction with Type I S.S.S. later than 2 months after recovery. Likewise, of the 6 cases reacting to Type III polysaccharide, only 3 were positive at the last test which, in 2 cases, was done less than

¹ In cases recovering by "lysis" it was found that the first day of the lysis period corresponded with respect to circulating antibodies to the day of "crisis," and it is so considered in the text and the accompanying figures.

2 weeks post-critically. In contrast to these results, only 1 of the 12 patients having Type II positive reactions failed to give a positive response at the time of the last observation. This suggests that the longer duration is not a characteristic of heterologous responses as such but, rather, a characteristic of the reactions to the Type II S.S.S. used.

Comparison of the Cutaneous Reactions and the Circulating Antibodies, and the Relation of Both to the Course of the Disease.—The time of

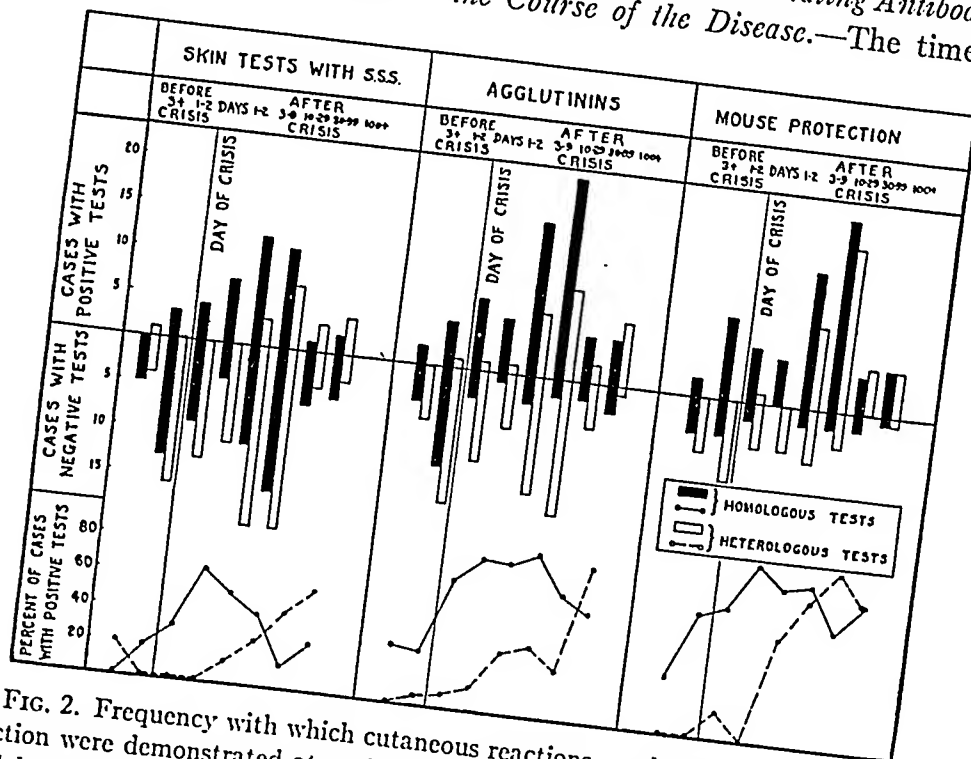


FIG. 2. Frequency with which cutaneous reactions, agglutinins and mouse protection were demonstrated at various stages of the disease in 30 recovered cases of lobar pneumonia receiving no specific therapy.

appearance of the skin reaction, of agglutinins and of protective antibodies for the homologous type could be compared in 24 cases. All 3 immune reactions were first demonstrated simultaneously in 5 cases; in 12 others, the protective antibodies and agglutinins appeared at the same time and before the skin reaction, when that was positive; and, in the remaining 7 cases, protective antibodies were demonstrated before either of the other reactions. Similarly, 19 cases could be compared

with respect to heterologous types. The skin reactions and the circulating antibodies were demonstrated simultaneously in 3 cases; protective antibodies and agglutinins were found at the same time and before the skin reaction in 6 cases; and, in 9 cases, protective antibodies appeared before either of the other reactions. In 1 case (J. McB., Type I), the appearance of the positive heterologous skin reaction preceded the finding of circulating antibodies. The frequency with which antibodies were found and positive skin reactions occurred at various intervals is represented graphically in Fig. 2.

When antibodies were present in any of the patients in this series they were demonstrated, in practically every instance, by the mouse protection test. The corresponding agglutinins were usually demonstrable, but this was not true in every case and often did not correspond at different times in the same case. All cases having positive cutaneous reactions with the type-specific polysaccharides had protective antibodies for the corresponding type of pneumococcus at the same time, but agglutinins were less regularly found. Figs. 3 to 6 show the results of skin tests and antibody determinations in 4 typical recovered patients.

From Fig. 2 it will be seen that agglutinins and protective antibodies, particularly the latter, appeared earlier than did the cutaneous reaction to the corresponding S.S.S., whether of the homologous or of the heterologous type. The persistence of heterologous antibodies over a period of over 3 months is again brought out rather strikingly.² The homologous agglutinins and protective antibodies persisted longer and in a larger percentage of cases than did the positive skin reactions with the corresponding polysaccharide.

Cutaneous Reactions and Circulating Specific Antibodies in Patients Tested on One Occasion

The results of the tests in patients tested once in convalescence and in those with no recent history of pneumonia are shown in Table II. It is seen that early in convalescence the cases which had previously received no skin tests frequently showed cutaneous reactions and anti-

² Two of the patients (S. R. and G. C. (Figs. 4 and 5)) were studied 12 and 13 months respectively after recovery. Cutaneous reactions and antibodies for more than 1 type were demonstrated in both of these patients.

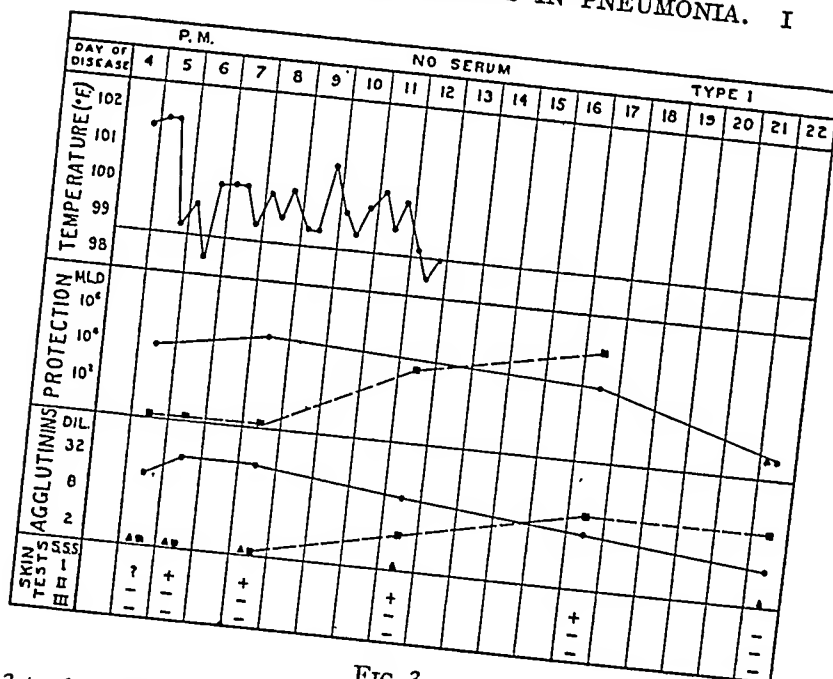


FIG. 3

FIGS. 3 to 6. Results of all of the tests performed in 4 different patients with lobar pneumonia receiving no serum therapy.

- — — — ● Type I tests.
- — — — ■ Type II tests.
- ▲ ▲ Type III tests.

+ Positive.
- Negative.
? Doubtful.

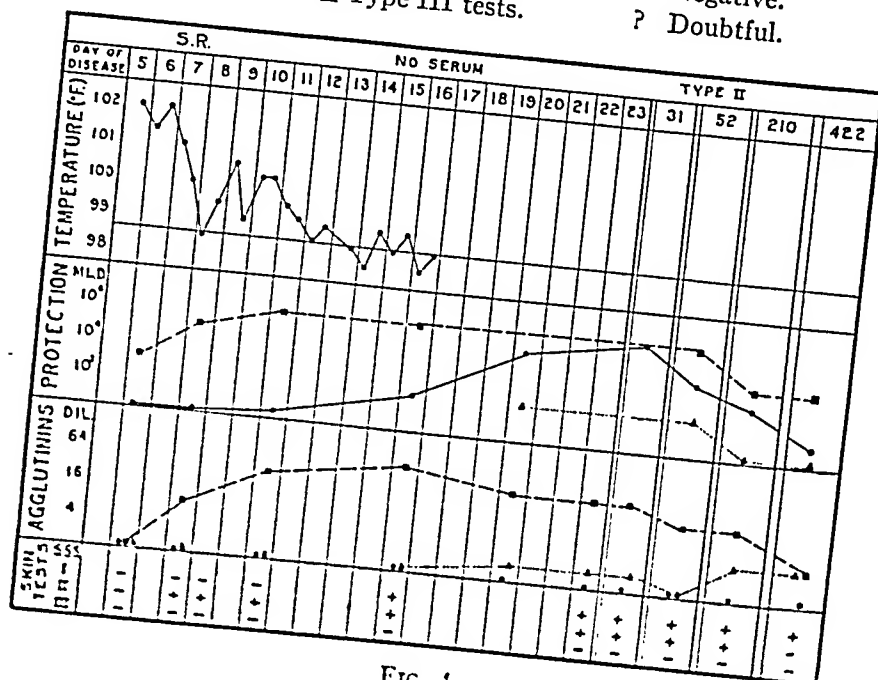


FIG. 4

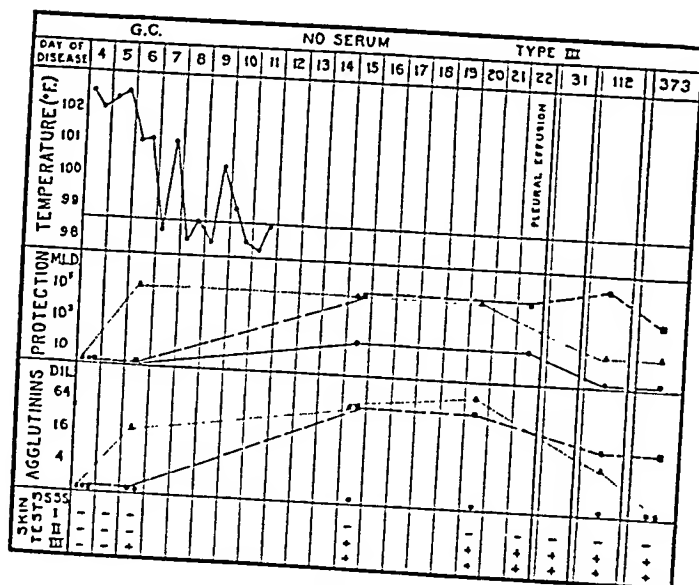


FIG. 5

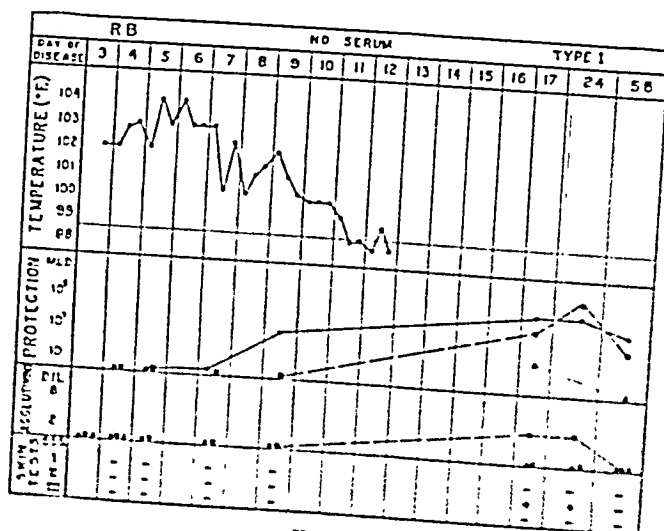


FIG. 6

REACTIONS AND ANTIBODIES IN PNEUMONIA. I

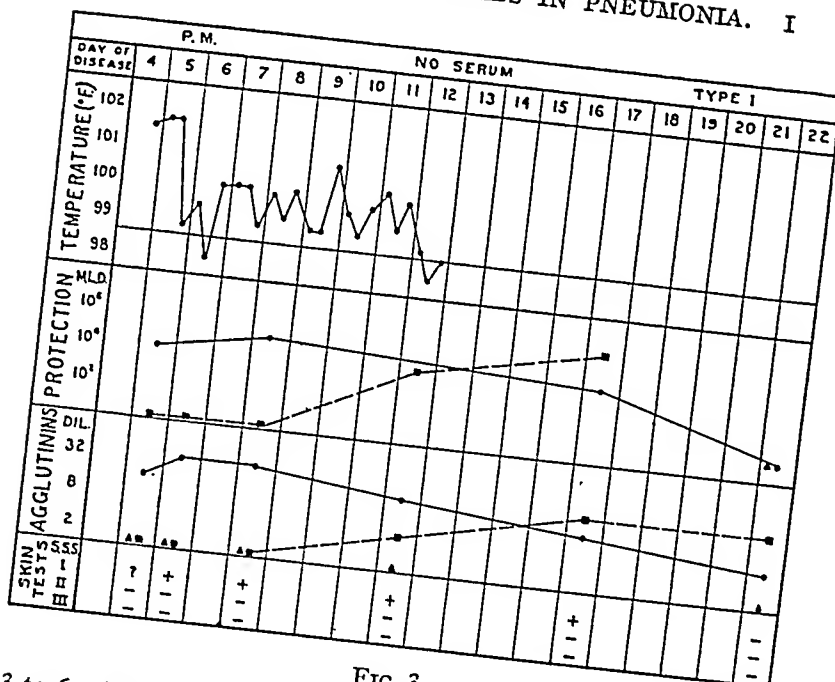


FIG. 3

FIGS. 3 to 6. Results of all of the tests performed in 4 different patients with lobar pneumonia receiving no serum therapy.

- — Type I tests.
- — Type II tests.
- ▲ Type III tests.

+ Positive.

— Negative.

? Doubtful.

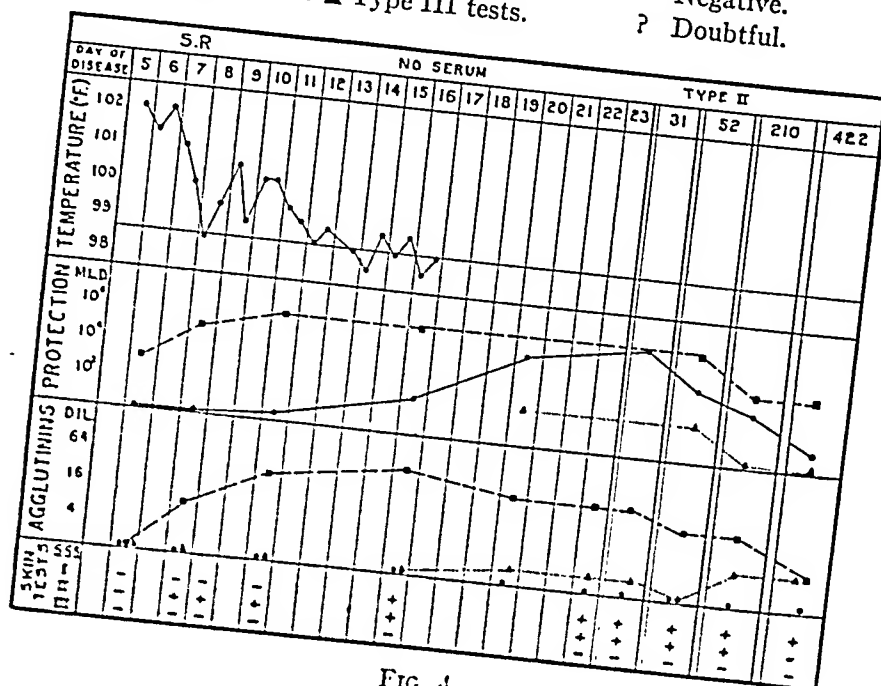
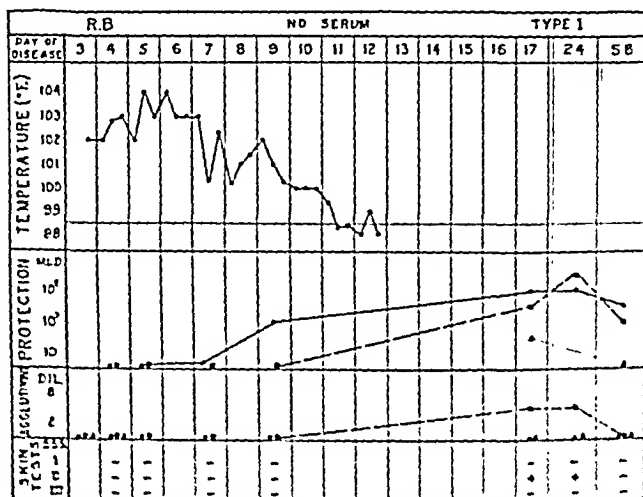
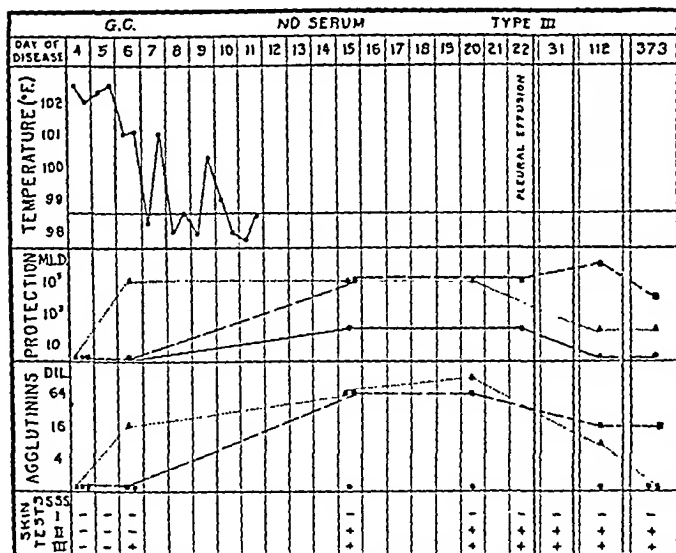


FIG. 4



bodies specific for the infecting type of pneumococcus. A few patients showed heterologous skin responses and antibodies, but these were no more frequent than in the group of cases with no recent pneumonia. In the few instances where heterologous circulating antibodies were found at the time of the skin test, these were also shown to have been present during the disease. In the cases tested 5 to 14 months after

TABLE II

Summary of the Cutaneous Reactions, Agglutinins and Protective Antibodies in 3 Groups of Cases Not Previously Tested Intradermally

	Type of infection	No. of cases	Positive cutaneous reactions			Agglutinins present			Protective antibodies present		
			Type I	Type II	Type III	Type I	Type II	Type III	Type I	Type II	Type III
<i>Group I</i>											
Tested 4 to 23 days after crisis	I	13	7	1	1	9	0	2	10*	0	1
	II	12	2	10	2	1	11	0	2	8	3
	III	4	0	3	1	0	0	2	0	0	2
	Miscellaneous pneumococci	6	2	2	0	1	1	0	1	1	0
<i>Group II</i>											
Tested 5 to 14 months after crisis	I	5	0	1	0	0	0	0	1	0	1
	II	2	0	1	0	0	0	0	0	1	1
	III	2	0	0	0	0	1	0	0	1	0
<i>Group III</i>											
Cases without recent pneumonia		24**	4	10†	0	0	0	0	0	5	1

* 2 additional cases had 10 M.L.D. and irregular survivals to 100 M.L.D.

** Protection tests done in 12 cases, 6 of which had positive skin tests.

† All of the cases having positive Type I tests also appear here.

recovery, typical positive cutaneous responses were often not associated with corresponding agglutinins or protective antibodies. This is in sharp contrast to the regular association of positive skin tests with corresponding antibodies in patients receiving repeated injections of S.S.S. The frequency of positive reactions in patients without recent pneumonia is also in sharp contrast to the almost complete absence of positive responses during the acute stage of the disease.

There were 5 patients with complications in the group tested early in convalescence.

One was a patient, who, 8 days after crisis, showed a positive cutaneous response to the Type I polysaccharide and circulating antibodies for the same type. On the same day, Type I pneumococci were cultured from pus obtained from the pleural cavity. The second patient was afebrile and apparently having a normal convalescence on the 9th day after crisis, at which time a positive Type I skin test was obtained and circulating specific antibodies for this type were present. 2 days later this patient had a rise in temperature, a positive blood culture was obtained and he died 2 days later. The organisms cultured from his blood, as well as those cultured at autopsy from the heart's blood, the involved lungs and pleural fluid, were Gram-positive, green-producing diplococci which were bile insoluble, grew in rough colonies and did not agglutinate with any of the specific sera available. This organism may have been a rough pneumococcus. 2 Type I patients had sterile pleural effusions; one of these had a positive homologous skin test. One other Type I patient in this group had an empyema; his skin tests were negative.

DISCUSSION

The observations of Tillett and Francis (1) that the protein-free, type-specific polysaccharide of the homologous pneumococcus elicits an immediate "wheal and erythema" type of reaction when injected into the skin of patients recovering from pneumococcus lobar pneumonia and that this reaction is associated with the presence of circulating specific antibodies has been confirmed. These authors found positive skin reactions in 100 per cent of 21 recovered Type I cases. In the present series only one-half of the Type I and two-thirds of the Type II and Type III cases that recovered showed skin reactions to the purified polysaccharide of the pneumococcus causing the disease, but these Type I cases are hardly comparable to those of Tillett and Francis, since most of their cases were treated with antipneumococcic serum.

The majority of fatal cases in this series failed to react to the homologous polysaccharides. Mention was made above of a patient who gave a characteristic skin response to the Type II S.S.S. at a time when he had massive blood invasion with Type II pneumococci. No mouse protective antibodies or agglutinins were found in this patient's blood serum. There is no adequate explanation for this peculiar occurrence, but two possibilities may be mentioned. In the first place, the antibody may have been fixed in the skin, although it had disappeared

from the blood stream. Secondly, this patient may have come in contact with some substances, such as yeasts (3), immunologically related to Type II polysaccharide, to which he had become partially immunized, and was therefore capable of reacting with the purified polysaccharide in the skin.

In the patients who had repeated skin tests with the specific pneumococcus polysaccharides antibodies were demonstrated to pneumococci heterologous to the type of infecting organism but corresponding to the types of polysaccharides used in the skin tests. Patients tested on only one occasion in convalescence did not show heterologous antibodies. Similar observations were recorded by Francis and Tillett (4) who suggested that these heterologous antibodies were associated with the previous intradermal injections. Direct experiments with normal individuals (5) have shown that the production of type-specific antibodies may be induced by single or repeated skin test doses of polysaccharides. It thus seems likely that the type-specific carbohydrates injected intradermally in small amounts are antigenic.

Although heterologous antibodies and sometimes heterologous skin tests developed for each of the 3 types in some of the cases, the heterologous serum antibodies and skin tests were by far most frequent for Type II. The explanation of this fact may depend on the properties of the solution of Type II S.S.S. used in this study which was possibly more actively antigenic than the Type I and Type III solutions. On the other hand, since "natural" antibodies for Type II pneumococci are present normally in nearly all adult human bloods (5), the basis for more effective immunization to Type II polysaccharide may be present in human subjects.

A study of Fig. 2 will indicate that the skin reaction is a less sensitive indicator of antibody production than is either the agglutination or mouse protection test. Furthermore, the large number of negative responses in patients who recovered without complications and the finding of positive tests in patients with persistent infection indicate the disadvantages of the skin test as a prognostic aid. The agglutination test, while demonstrably somewhat less frequently than mouse protection and, thus, a less delicate index of antibody formation, has the advantages of simplicity, of the absence of irregularities so often presented by the use of the mouse and of rapidity. When performed by

such methods as those of Arlyle Noble (6) and of Sabin (7), the agglutination reaction is as simple to perform as the skin reaction and is less time consuming.

SUMMARY AND CONCLUSIONS

1. A group of 41 non-serum treated patients with Type I, II or III pneumococcus pneumonia were studied during their disease and convalescence with respect to their skin reactions to specific pneumococcus polysaccharides and, in most instances, for the presence of circulating agglutinins and protective antibodies for all these 3 types.

2. One-half of the Type I and two-thirds of the Type II and Type III recovered cases gave the typical immediate "wheal and erythema" response to the homologous polysaccharide at or about the time of recovery. All cases tested showed protective antibodies and almost all showed agglutinins for the homologous pneumococcus. In the fatal cases, in general, positive cutaneous reactions and circulating antibodies were not obtained.

3. In cases of pneumonia receiving repeated cutaneous inoculations with various types of specific polysaccharide, antibodies for pneumococci differing from the infecting type but corresponding to the types of carbohydrate injected were present 1 week or later after such injections. These heterologous antibodies were most frequently demonstrated for Type II and were probably the result of immunization by means of the cutaneous injections.

4. Positive skin responses to homologous polysaccharides and corresponding circulating antibodies were demonstrated with similar frequency in the first 3 weeks after crisis in patients who had not previously received intracutaneous injections. In such patients heterologous antibodies were rarely found.

5. Typical skin reactions with the specific pneumococcus polysaccharides and mouse protective antibodies were demonstrated independently in a number of hospital patients who had had no recent history of pneumonia.

6. Some patients with demonstrable foci of persistent infection or with latent infections which later proved fatal showed positive cutaneous responses to the homologous type polysaccharide and circulating specific antibodies for the corresponding type.

peared immediately after the injection of this amount. The reaction was again elicited after 6 hours, but not after 30 hours. Some lots of serum failed to give this response with the same or larger amounts. It was also found possible in cases of lobar pneumonia to elicit positive cutaneous responses within a few minutes after the injection of various amounts of specific antisera.

Materials and Methods

The patients upon whom these studies were made were, in general, similar to those used in the previous study (1) except that the present cases were treated with specific antipneumococcic sera. In all, 31 cases were studied. Felton's bivalent (Types I and II) concentrated antibody solution was given to 17 Type I and 11 Type II cases; 3 of the former and 2 of the latter terminated fatally. Convalescent sera from homologous type cases were given to 1 Type II patient and to 2 patients with Type III pneumonia. All 3 of these patients died. The age of the patients ranged from 13 to 76 years, 4 being under 20 and 2 over 60. All but 4 were males. Only 3 of the 28 recipients of Felton's concentrated antibody solution had a positive blood culture (J. O'B., Type I, and B. M., Type II, (Fig. 1) and a fatal Type II case) whereas all 3 of the convalescent serum recipients showed pneumococci in their blood. Serum administration was begun in these cases from 36 hours to the 8th day after the onset of the disease and all were acutely ill at the time the first dose was given.

Skin tests were performed and agglutinins and protective antibodies determined as described in a previous paper (1). Control skin tests with the polysaccharides were done and samples of the patients' blood were obtained 30 minutes or more before the first injection of serum. Subsequently, skin tests were performed and blood samples were obtained before further injections of serum and at intervals during convalescence.

Immune Sera.—A description of the materials and methods employed in the administration of serum to the Type I cases is given elsewhere (2). The concentrated sera used in these cases was furnished by the Antitoxin and Vaccine Laboratory of the Massachusetts Department of Public Health through the courtesy of Dr. Benjamin White. The concentrated antibody solution used in the treatment of the Type II cases of this series was prepared and supplied by Dr. Felton (3) of the Department of Preventive Medicine and Hygiene of the Harvard Medical School and contained 2000 to 3000 units (4) of mouse protection per cubic centimeter against Types I and II pneumococci. The convalescent serum recipients were studied in the same manner as the other patients. The methods used for preparing and giving the convalescent sera in these cases are included in the clinical report by Beebe and Sutliff (5). Skin tests were done on the donors at the time that their blood was obtained, and their sera were tested for antibodies.

Varieties of Cutaneous Response in Serum Treated Cases.—All the various degrees

of response observed in the cases receiving no specific therapy (1) were seen in this group except that reactions with edema of several hours duration were less common. Skin tests which showed no reaction for 30 minutes or more after injection, or skin tests, which, once positive, had already faded, sometimes gave a typical response within a few minutes following a subsequent dose of serum. In one instance (J. K., Type II), only the last of 7 skin tests done at 1 to 2 hourly intervals during the 1st day of treatment showed a positive Type II response. The next skin test performed 16 hours later was positive with Types I and II S.S.S., and was followed, 2 minutes after the injection of the polysaccharide, by the appearance of blotchy wheals and erythema formations at the site of all previous injections of Types I and II S.S.S.

Results in Cases of Type I and Type II Pneumonia Treated with Bivalent (Types I and II) Concentrated Antibody Solution (Felton)

First Appearance of Positive Cutaneous Reactions.—Observations were made before treatment in 20 of the 28 patients receiving Felton's serum. In none was a positive response elicited to any of the 3 polysaccharides injected. The first skin test after the beginning of serum therapy was positive with the carbohydrate homologous to that of the disease in 14 of the 23 recovered cases. This positive response was elicited in 10 of these 14 cases within 24 hours after the first dose of serum was given. Of the 9 recovered cases whose first test after the institution of serum treatment was negative, 5 gave a positive homologous response within 11 hours after the 1st dose, in the 6th the second test done the next day was positive, and in the 3 remaining patients a positive homologous response was never elicited.

The reaction to both the Type I and the Type II polysaccharides usually became positive at the same interval after serum administration regardless of the type of the infecting pneumococcus. The few exceptions may be seen by a study of Fig. 1 on which are represented graphically the results of all the skin tests done in the recovered cases treated with the concentrated antibodies.

Relationship between the First Appearance of the Positive Cutaneous Response and the Amount of Serum Administered.—A positive response to the homologous S.S.S. was elicited in 10 of 13 patients tested after receiving amounts of serum up to 40 cc., and in 8 other patients at the time of the first test after the injection of serum, when more than 40 cc. had already been given. 2 cases that showed doubtful or negative

responses after small doses showed positive reactions after 93 and 85 cc., respectively. The frequency with which positive skin tests were obtained after various doses of serum and at various intervals after its administration is shown graphically in Fig. 2.

Duration of the Skin Reactivity in Serum Treated Patients.—The frequency with which positive tests were elicited diminished rapidly

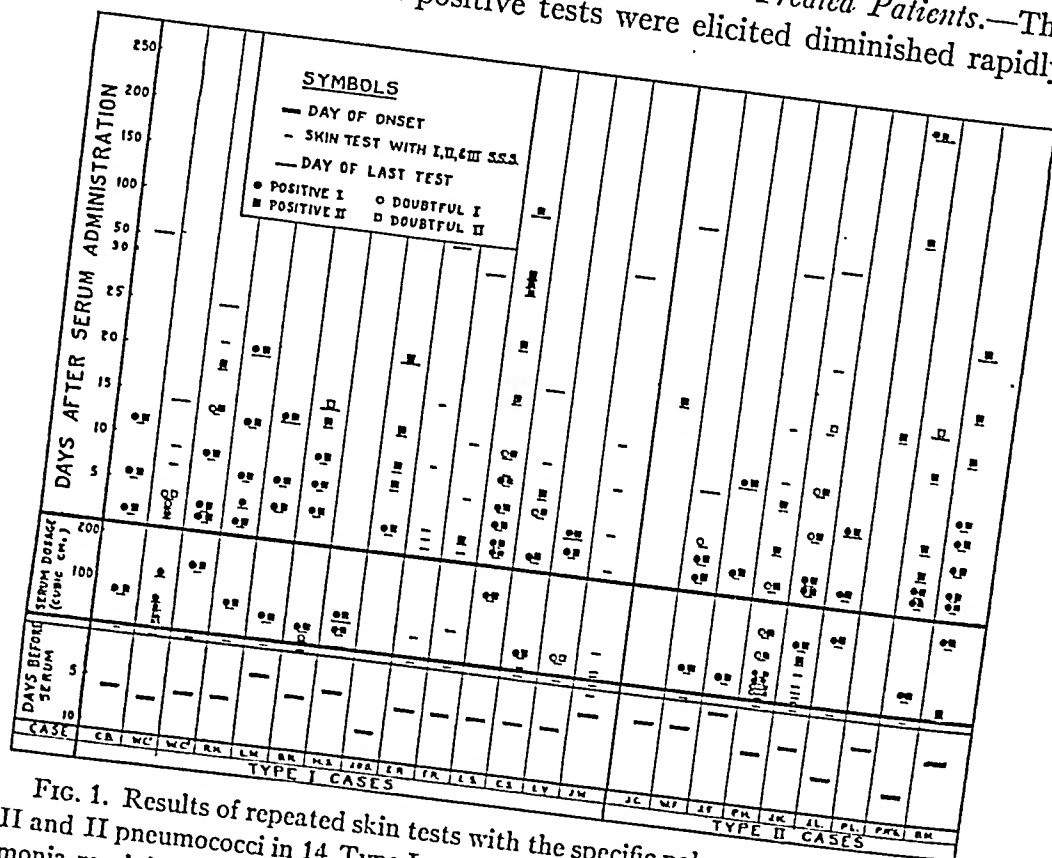


FIG. 1. Results of repeated skin tests with the specific polysaccharide of Type I, II and II pneumococci in 14 Type I and 9 Type II recovered cases of lobar pneumonia receiving bivalent (Type I and II) concentrated pneumococcic antibodies (Felton).

after the 3rd day following serum administration, as shown graphically in Fig. 2. Only 2 of 9 cases in this series on whom tests were done later than 1 month after recovery gave positive cutaneous responses to S.S.S.: a Type I case on whom a positive Type II test was elicited at the end of the 4th month and a Type II case on whom both Type I and Type II S.S.S. gave positive reactions more than 8 months after

serum treatment. This diminution in the frequency of positive tests as convalescence progressed was more striking for the reactions elicited by the Type I S.S.S. than for those obtained with the Type II carbohydrate, regardless of whether the infecting organism was Type I or II.

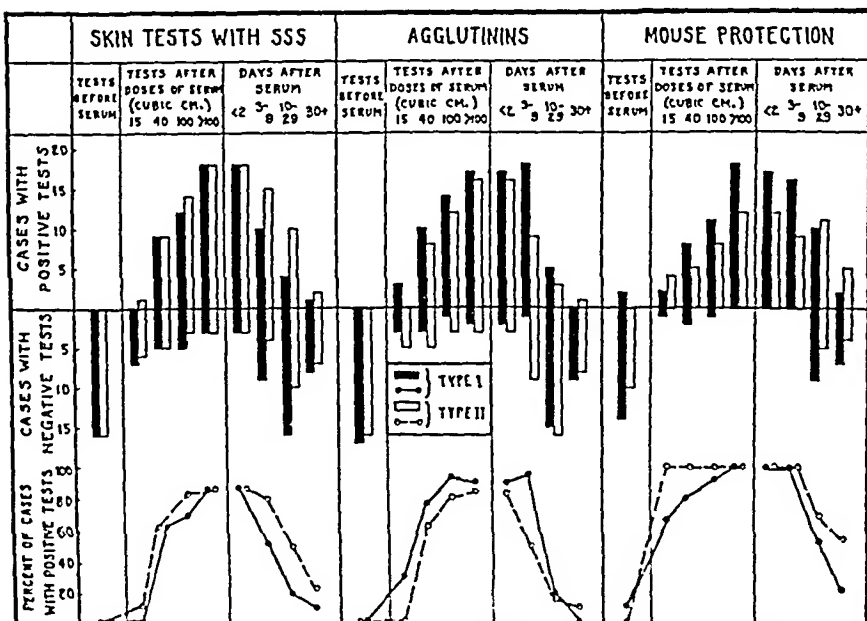


FIG. 2. Frequency of cutaneous reactions, agglutinins and mouse protection at various intervals in 23 recovered cases of lobar pneumonia treated with bivalent (Type I and II) concentrated pneumococcic antibodies (Felton).

Skin Tests in Fatal Cases and in Cases Recovering with Complications.—A positive test was elicited in only 1 of 5 fatal cases receiving concentrated antibodies.

This was obtained in a Type II patient with the homologous S.S.S. 48 hours after the last dose of serum was given and 20 hours before the patient's death. One of the fatal cases, from whose sputum Type I pneumococci were recovered at the time of admission to the hospital, was given a total of 335 cc. of concentrated serum in repeated doses over a period of a week and failed to give a positive response to any of the cutaneous tests. Blood cultures in this case were repeatedly negative.

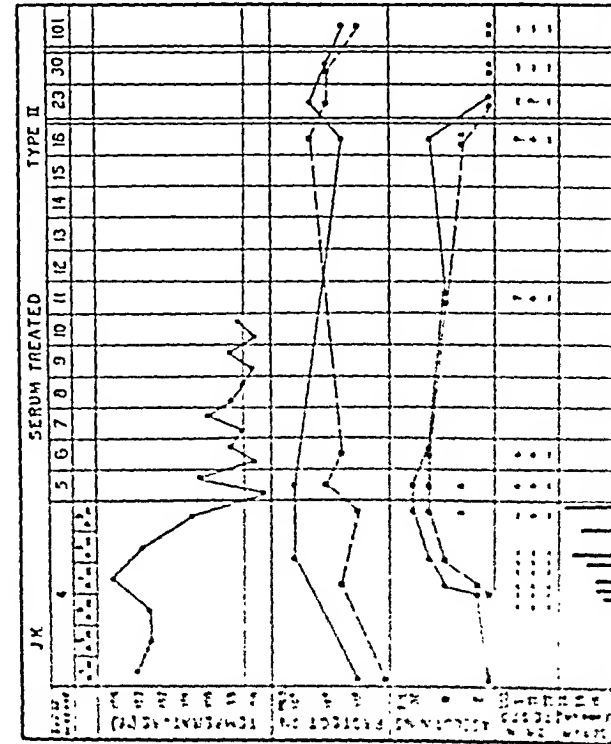


FIG. 5

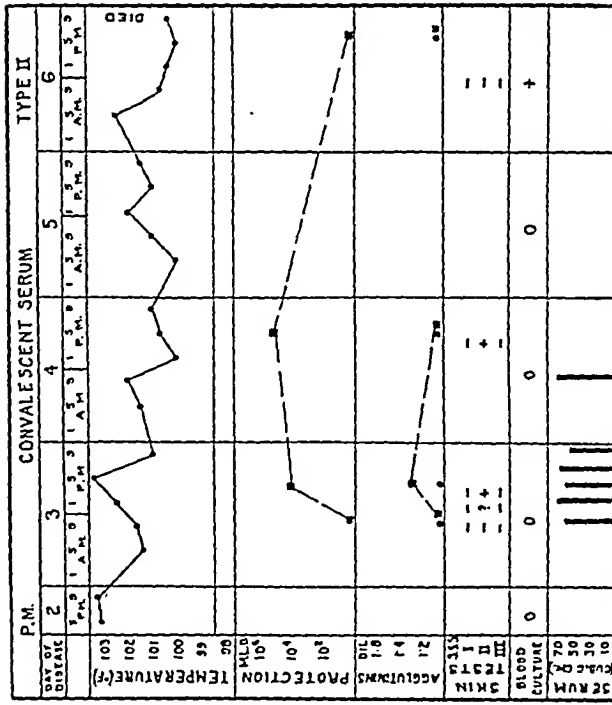


FIG. 6

Cultures at autopsy from each of the affected lobes of the lung, from several abscesses in these lobes and from both parotid glands, which were also abscessed, showed pure cultures of *Staphylococcus aureus*, but no pneumococci.

There was only 1 case in the series that developed a purulent complication (J.O'B., Type I, see Fig. 1). This patient had positive Type I and II tests on the 2nd day after serum was given, but 5 days later the Type I test was negative. He had a purulent infection of the left shoulder joint from which Type I pneumococci were recovered. 2 patients with sterile pleural effusions (W. C. and L. S., Type I) showed the usual positive responses.

Comparison of the Skin Response and Circulating Antibodies in Serum Treated Cases.—Agglutinins were studied in the sera of almost all patients for all 3 types before and after serum administration. Tests for protective antibodies against Types I and II were carried out in 17 patients and for the homologous type alone in 7 other cases. None of the patients tested showed agglutinins for any of the types before the onset of serum therapy. In only 2 patients (J. K., Type II, and F. R., Type I) were protective antibodies (against 100 lethal doses of Type I in each case) found before treatment. Fig. 2 shows graphically the frequency with which cutaneous reactions, agglutinins and protective antibodies were demonstrated at various intervals.

In 7 cases in which the antibodies were not first demonstrated simultaneously, protective antibodies tended to appear first or at the same time that agglutinins were demonstrated, and the positive cutaneous responses, were subsequently elicited. Later in the disease, the order in which the antibodies disappeared was not as regular as in the cases receiving no serum. In general, protective antibodies tended to persist longer than agglutinins or skin reactivity, but 2 instances (R. H., Type I, and B. M., Type II) were observed in which positive skin reactions were obtained without protective antibodies or agglutinins for the corresponding type. There was but one instance in which agglutinins were present in the absence of corresponding protective antibodies. The sera from many cases had protective antibodies without corresponding agglutinins. 5 cases were shown to have protective antibodies 2 months or more after recovery; all of these had Type II protection, and 2 had, in addition, Type I protection (J. K., Type II, and F. R., Type I).

The results of all the tests in 3 typical patients are shown graphically in Figs. 3 to 5.

Variations in the Skin Response Following the Administration of Various Lots of Serum.—The data on this subject are necessarily meager inasmuch as several lots of serum were used and only a small number of cases treated with each lot. That different lots may vary in their effect on the production of positive skin tests is suggested by experiences with 2 lots.

Lot CP₇, containing 3500 units of mouse protection against Type I and 1500 units against Type II, was given to 4 Type I patients and 1 Type II patient all of whom recovered. Positive tests with Types I and II S.S.S. were obtained with the first test following serum administration in 4 patients, only 1 of whom had received more than 35 cc. at that time. One Type I case had a positive reaction to Type I S.S.S. after 35 cc., but a positive Type II test was never obtained. Lot CP₁₃, containing 3000 units against Type I and 1500 against Type II, was given to 3 Type I patients. 2 patients received 45 and 75 cc., respectively, yet failed to give positive reactions to either Type I or Type II S.S.S. The 3rd patient is not included in this series, as complete immunological studies were not made. He was treated with 180 cc. of the material on the 4th day of the disease, but failed to show any clinical improvement from the serum and died within 24 hours.

Cutaneous Reactions in Cases of Type II and Type III Lobar Pneumonia Treated with Homologous Human Convalescent Serum

3 patients received convalescent sera obtained from patients about 1 week after recovery from the homologous type lobar pneumonia. All the donors gave positive cutaneous reactions to the corresponding type S.S.S., and had demonstrable agglutinins and mouse protective antibodies in their sera.

One of the recipients (P.M., Fig. 6), a Type II case who had negative skin tests, no demonstrable circulating antibodies and a negative blood culture before serum was given, showed a positive homologous skin test 1 hour after 150 cc. of serum was given. At this time he also had demonstrable protective antibodies and agglutinins for Type II pneumococci in his serum. 2 days later, no further serum having been given, his blood culture became positive for the first time, his skin tests were negative and no circulating antibodies were demonstrable, and the patient died. 2 other patients, with Type III infection, received 75 and 310 cc. of serum, respectively, but in neither was a positive response to Type III S.S.S. elicited, nor were circulating antibodies demonstrated on repeated tests. Both of these cases also ended fatally. The patient receiving the larger dose had a steadily increasing

bacteremia in spite of the repeated doses of serum. He had, before serum, a strongly positive skin reaction to Type II S.S.S., and protection against 100 lethal doses of Type II pneumococci was demonstrated in his serum at that time. The Type II positive skin reaction was repeatedly elicited and remained strongly positive until 4 hours before death, at which time no wheal appeared but erythema of the same extent, but less intense, resulted. Type II agglutinins were not found and Type II protective antibodies were not demonstrated after serum was given.

TABLE I
Cutaneous Reactions with S.S.S. and Circulating Specific Antibodies in 3 Groups of Cases Treated with Felton's Serum

		No. of cases	Positive skin tests			Protection present			Agglutinins present		
			Type I	Type II	Type III	Type I	Type II	Type III	Type I	Type II	Type III
Tests performed 10 to 22 days after serum administration	Cases previously tested with S.S.S.	19*	4	12	0	11	12	—	8	6	0
	Cases not previously tested	9**	5	6	0	9	8	—	5	2	0
Tests 5 to 14 mos. after serum	No previous tests	11†	4	6	0	0	0	0	0	0	0

— No tests done.

* Type II protection tests were done in only 15 of these cases.

** One of the cases of empyema had positive reactions to Types I and II S.S.S. † Protection tests were done in only 5 patients, all of whom had positive skin tests. The 4 patients having positive Type I reactions had positive Type II skin tests as well.

Single Skin Tests and Antibody Studies after Serum Administration

It has previously been shown that the intracutaneous injection of the type-specific pneumococcus polysaccharides may result in the production of antibodies specific for the type of carbohydrate injected (1, 6). In order to learn whether this factor played a significant rôle in the serum treated cases discussed above, 20 cases receiving Felton's serum were studied at a single occasion after serum had been given.

These cases may be divided into 2 groups. The first group was studied for skin reactions and specific antibodies 10 to 22 days after serum administration. This group included 6 cases having Type I pneumonia (2 of these had empyema at the time they were studied),

2 with Type II and 1 with a miscellaneous pneumococcus infection. The second group was studied 5 to 14 months after recovery. This group consisted of 11 Type I recipients of Felton's serum, including 2 patients who had empyema and 2 others who had sterile pleural effusions following their disease. The number of cases showing positive tests in these 2 groups are shown in Table I. In this table is also included a group of the cases previously described that received repeated skin tests and were again tested from 10 to 22 days after serum.

In the group of cases tested for the first time during the 2nd or 3rd week after serum administration the incidence of positive skin reactions, of agglutinins and of protective antibodies is not significantly different from that found during the same period in the serum treated cases who had previously received several skin tests with the specific carbohydrates.

DISCUSSION

The observations here presented demonstrate the possibility of "producing" and maintaining for some time a positive response in the human subject to the cutaneous injection of the purified, protein-free, type-specific carbohydrate of the pneumococcus. This is accomplished by the intravenous administration of the corresponding immune serum derived either from the horse or from the human patient after recovery from lobar pneumonia. Previous observations by Cole (7), and more recently by Sutliff (8), and by Lord and Persons (9), have indicated that demonstrable agglutinins and protective antibodies may be produced and maintained in the blood of patients with lobar pneumonia by the administration of sufficient amounts of potent immune sera. These observations are here confirmed and extended to include the specific cutaneous response to the pneumococcus polysaccharides.

The purified type-specific polysaccharide, although only a part of the pneumococcus antigen, reacts specifically with antibodies produced by the whole antigen (10, 11). Tillett and Francis (12) have further observed that the carbohydrate may induce a reaction in the skin of patients recovering from lobar pneumonia and that this reaction is specific for the type of infecting pneumococcus and is associated with the presence of agglutinins and precipitins in the sera of the patients. Attempts to stimulate the production of antibodies by the purified carbohydrates have, however, failed (11, 13). These substances have,

therefore, been considered non-antigenic and the term "haptenes" applied to them by Landsteiner (14, 15), has been generally accepted. More recently, however, it has been shown by Francis and Tillett (6) and in a previous communication (1) that these purified carbohydrates injected in minute amounts into the skin of patients ill with or recovering from lobar pneumonia and receiving no specific antisera are capable of stimulating the production of antibodies specific for the type of carbohydrate injected. Further observations (16) have shown that this is possible in normal individuals, suggesting the antigenic character of these bacterial carbohydrates when used in this manner.

The duration of the immune reactions (Fig. 2) in serum treated patients receiving repeated intracutaneous injections of polysaccharides appears to be distinctly shorter than in the corresponding patients previously reported (1). In the former group such reactions were rarely found 1 month after recovery, whereas in the latter homologous and heterologous antibodies persisted for several months.

These 2 groups of cases were in every respect comparable except for serum administration. It may thus be inferred that the passive introduction of antibodies in patients receiving specific polysaccharides intracutaneously interferes with antibody production of the carbohydrates. This is similar to the well recognized failure of individuals to develop effective active immunity in other diseases following the intensive therapeutic use of antibacterial or antitoxic sera.

The results here presented show that, in most instances, cases terminating fatally after the administration of specific serum fail to give positive skin responses. This failure to react apparently cannot always be interpreted as indicating a persistence of the type-specific pneumococcus infection. That the failure of these fatal cases to react may be non-specific is suggested by the absence of positive reactions to the polysaccharides corresponding to the heterologous antibodies injected and by the failure of one patient to give a positive response to either the Type I or the Type II polysaccharide although he had a massive infection with *Staphylococcus aureus* and pneumococci could not be recovered from his organs at postmortem. Inhibition of heterologous skin reactions in other acute infections has been reported by others (17). The almost complete absence of cutaneous reactions in these individuals is probably not due to the inability of the patients'

skin to react, for typical reactions to the Type II polysaccharides occurred in 3 cases terminating fatally (1 of these is reported in a previous paper (1)) and such patients were, in some instances, shown to respond to small doses of histamine with the characteristic wheal and erythema.

Cases which failed to give characteristic positive reactions did not all terminate fatally. The failure to induce positive cutaneous responses by the injection of antisera in patients, in whom clinical benefit is otherwise obvious, may depend on the character of the serum, some sera being more or less deficient in the quality of inducing cutaneous responses, or on the responsiveness of the patient. Similar lack of reactivity in some normal individuals was observed by Coca and Grove (18) in connection with the passive transfer of atopic reagins.

The presence of foci of infection has not been constantly associated with the absence of skin responses. About one-half of the surviving patients with well established foci of pneumococcus infection persisting after recovery from pneumonia, both with and without serum treatment, showed cutaneous responses after these foci were demonstrated.

To attempt to define the curative dose of antipneumococcic sera on the basis of immune reactions in the patient is hazardous. The general correlations, however, between the occurrence of positive skin reactions and recovery, and between the dosage of immune sera and the appearance of the positive skin reaction are sufficiently regular to be at least suggestive. In the majority of the patients in this series who recovered and showed positive reactions, such reactions first appeared and were maintained after doses of 40 cc. of Felton's concentrated antibodies. It is thus suggested that the effective curative dose of most of the preparations used, perhaps only in the milder cases, is in the vicinity of this amount.

The relationship of the skin test to agglutinins and protective antibodies has been discussed in a previous communication (1) and nothing further need be added here.

SUMMARY AND CONCLUSIONS

1. Characteristic cutaneous responses to the type-specific protein-free carbohydrates of both Type I and Type II pneumococci have been

"produced" in cases of lobar pneumonia due to either of these types by the intravenous injection of concentrated bivalent (Types I and II) antipneumococcic sera (Felton).

2. A positive cutaneous response to the specific polysaccharide of Type II pneumococci has been passively transferred from human cases convalescing from this infection to a patient suffering from pneumonia due to this organism.

3. The cutaneous responses to the type-specific polysaccharides and circulating antibodies were studied in 51 cases of lobar pneumonia. Positive cutaneous reactions were, in most instances, associated with recovery, even when purulent complications were present. Failure to elicit a positive reaction was usually followed by a fatal outcome.

4. The positive reactions in patients who were treated with concentrated sera and recovered were most often elicited within 24 hours after the first dose and after a total of 40 cc. had been given.

5. The positive skin reactions obtained after the administration of specific antisera were associated with the presence of mouse protective antibodies and agglutinins in the sera of the patients.

6. The immune reactions in serum treated cases receiving repeated inoculations with the specific carbohydrates disappeared more rapidly than in similar cases receiving no antiserum. It is suggested that the administration of antisera in some way interferes with the production of antibodies by the intracutaneously injected carbohydrates.

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ARTIFICIAL ACIDOSIS IN TRYPANOSOMA LEWISI INFECTIONS, AND ITS BEARING ON THE PATHOGENIC ACTION OF TRYPANOSOMA EQUIPERDUM

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Recent studies on biochemical changes in animals experimentally infected with trypanosomes have clearly demonstrated that disturbances in the acid-base equilibrium of the blood are of fundamental significance in the pathogenic action of these organisms.

Kligler, Geiger, and Comaroff (1), working with *T. evansi* in rats, found that the lactic acid content of the blood increased progressively as the number of trypanosomes increased, until before death it stood at a figure three or four times normal. Linton (2), who used *T. equiperdum* in his experiments, determined the CO₂-combining capacity of the serum throughout the disease in rats. Beginning at a relatively early stage, the alkali reserve was lowered, and a moderate to severe acidosis was present. There was no regularly greater depletion of the alkali reserve in the later stages of the disease. On the contrary, the variation between different animals was considerable. Andrews, Johnson, and Dormal (3), who also worked with *T. equiperdum*, found similarly that "the trypanosome-infected rats suffered for some time before their demise with a non-volatile acidosis." The average figures for the CO₂-combining capacities derived from their experimental animals led them to believe, however, that the acidosis was confined to the later stages of the disease. Their results also indicated that the pH of the blood was abnormally low at death.

Linton (4) has compared the biochemical changes in fatal trypanosome infections and in non-fatal ones due to *T. lewisi*. He showed that the CO₂-combining capacity remained normal in animals infected with *T. lewisi*. While a given concentration of *T. equiperdum* per cubic millimeter caused a definite lowering of the CO₂-combining capacity, a like concentration of *T. lewisi* was unaccompanied by any change in the alkali reserve. The ability of *T. equiperdum* to affect the host adversely in respect to the alkali reserve was not, therefore, due to the numbers

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of the organism, but to some peculiarity in its metabolism, the result of which was to reduce the CO_2 -combining capacity. In this conclusion he differed from the opinion expressed by Andrews and his collaborators, who have invoked a mechanical cause for the lethal effect of *T. equiperdum*. As will be pointed out in the discussion, these two views are not mutually exclusive.

In view of what has been ascertained in regard to acid-base changes in infections with pathogenic trypanosomes, it seemed of interest to study the effect of an artificially induced acidosis upon infections with *T. lewisi*, thus reproducing for this organism a chemical environment in the blood somewhat similar to that in which other trypanosomes exhibit such a high degree of virulence.

Technique

The alkali reserve in rats was lowered by the intraperitoneal injection of ammonium chloride in 2 per cent solution. The solution was freshly made at intervals of 2 or 3 days as it is easily contaminated with fungi. Ammonium chloride has been employed by a number of workers to produce an acidosis. The literature on its use has been reviewed by Seegal (5), who also discusses the probable manner in which it acts to increase the hydrochloric acid metabolism. Varying amounts of this solution were given in doses of 0.25 cc., usually at intervals of 20 minutes, and the trypanosomes were counted at intervals during and after the course of injections. Dyspnea was always present in the animals after two or three injections, and was the chief symptom, although some of the animals showed considerable weakness. All recovered perfectly within a few hours after the last injection.

The CO_2 -combining capacity of normal rats lies within the same range of variation as that of man, that is, from 55 to 75 volumes per cent. To test the effect of the injection of ammonium chloride on normal animals, nine rats were given four injections of the 2 per cent solution at intervals of 20 minutes. At the end of the hour they were anesthetized with sodium amytal, bled from the heart at intervals from 10 minutes to 4 hours, and the CO_2 -combining capacities of their sera determined. The results are shown in Table I. As would be expected from determinations on a series of animals, instead of on a single one, the figures obtained are not absolute functions of the elapsed time. Nevertheless, in each of the first seven animals a moderate acidosis was present. In the last two the CO_2 -combining capacities had returned to normal, indicating the restoration of the buffers to their proper concentrations. Sodium amytal itself does not affect the CO_2 capacity,

as previous work (4) has shown. This experiment served to indicate the time limits within which the ammonium chloride could be expected to affect the alkali reserve, and these limits were taken into account in the experiments.

Table II gives complete protocols for two of the experimental animals (Rats 6 and 11), to illustrate the general plan according to which the experiments were carried out. As the protocols show, the trypanosomes were first counted, the rats were then given two or three injections of the ammonium chloride solution, and the concentration

TABLE I

Carbon Dioxide-Combining Capacities of the Sera of Normal Rats after the Intra-peritoneal Injection of 1 Cc. of a 2 Per Cent Solution of Ammonium Chloride in 0.25 Cc. Amounts at 20 Minute Intervals

Rat No.	Interval after final injection	CO ₂ capacity
	<i>min.</i>	<i>vol. per cent</i>
1	10	51.2
2	15	49.3
3	20	51.2
4	20	41.5
5	40	35.9
6	50	52.1
	<i>hrs.</i>	
7	1½	39.6
8	3½	62.8
9	4	61.1

of trypanosomes again determined. The counts were made on blood from the tail vein. It was found that the effect of ammonium chloride was cumulative to a certain degree, and accordingly as the experiment progressed the doses were given at less frequent intervals.

Rat 11 was given 1.5 cc. of ammonium chloride in six doses during a period just under 4 hours. The final trypanosome count was made at the end of 5 hours and showed that the parasites had increased 300 per cent over the preliminary count. In Rat 6, which had a total of 1.0 cc. of ammonium chloride in four injections during an hour and a quarter, the parasites increased 146 per cent in the 2½ hours of the experiment.

ARTIFICIAL ACIDOSIS IN *T. LEWISI* INFECTIONS

Table III summarizes all of the experiments, but gives only the total increases and decreases of trypanosomes, the percentages changes, and the total amount of ammonium chloride injected. It also includes, as controls, a number of rats with *T. lewisi* infections, but without injections of ammonium chloride. The data are arranged according to the number of parasites per cubic millimeter, both before and after the

TABLE II
General Plan of the Experiments, Illustrated by Complete Protocols of Two Animals

Rat No.	Hour	Trypanosomes	Amount NH_4Cl injected	Percentage increase	Total percentage increase
11	<i>p.m.</i>	<i>per c.mm.</i>	<i>cc.</i>		
	12:30	11,950			
	12:45				
	1:05		0.25		
	1:25		0.25		
	1:50	20,050	0.25		
	2:00			66	
	2:55	27,500	0.25		
	3:15			37	127
	4:20	35,800	0.25		
	4:40			30	200
	5:45	53,300	0.25		
Total NH_4Cl given: 1.5 cc.; time: 5½ hrs.				47	340
6	1:20	71,000			
	1:30				
	1:50		0.25		
	2:10	121,500	0.25		
	2:15			70	
	2:45	139,150	0.25		
	2:45			14	95
	4:00	174,800	0.25		
Total NH_4Cl given: 1.0 cc.; time: 2¾ hrs.				25	146

crisis. Inspection of Table III shows: (1) that the injection of ammonium chloride in the first period of the infection causes a marked and rapid rise in the number of trypanosomes; (2) that in the second and third periods on the ascending curve the increase is progressively less marked, and closer to that shown by the controls, although it is still considerably greater than the control increases; (3) that near the

crisis the acidosis is without effect in increasing the number of trypanosomes. This point is illustrated by Rat 17, which was given 1.5

TABLE III

Percentage Change in Numbers of T. lewisi Per Cubic Millimeter, in Animals with and without Injections of Ammonium Chloride

Rat No.	Variation in thousands From To	Percentage increase Ante crisis	Hours of observation	Amount of NH ₄ Cl injected
				cc.
1. Control	14 - 21	50	4	0
2. Acidosis	12 - 53	340	5½	1.5
3. Acidosis	11 - 53	380	5½	1.0
4. Acidosis	14 - 43	270	4½	1.5
5. Control	31 - 40	29	4	0
6. Control	56 - 68	21	4	0
7. Acidosis	25 - 112	348	7	1.5
8. Acidosis	40 - 114	185	4½	1.0
9. Acidosis	71 - 175	146	2½	1.0
10. Control	117 - 129	10	3½	0
11. Control	155 - 161	4	4½	0
12. Acidosis	110 - 136	23	2	0.75
13. Acidosis	164 - 213	30	4½	0.75
14. Acidosis	123 - 187	52	5½	1.5
15. Control	174 - 185	6	4½	0
16. Acidosis	195 - 224	14	3½	1.25
17. Acidosis	187 - 201	7	8½	1.5
18. Acidosis	201 - 215	7	4½	1.5
		Percentage decrease Post crisis		
19. Control	155 - 151	2	4	0
20. Control	98 - 90	8	4	0
21. Control	66 - 59	10	4½	0
22. Acidosis	213 - 203	5	1½	0.75
23. Acidosis	101 - 94	6	2	0.75
24. Acidosis	69 - 66	4	1½	0.75
25. Acidosis	200 - 172	9	1½	0.75
26. Acidosis	59 - 52	12	6	1.75

cc. of ammonium chloride and observed over a period of 8½ hours; it showed, however, an increase of only 7 per cent during this period;

(4) that after the trypanosomes have begun to disappear from the blood, the injection of ammonium chloride has no influence upon their numbers, either to increase or decrease them. In each case a decrease was noted, and the percentage value found was of the same order as in the controls.

It is clear from this table that the injection of ammonium chloride does not change the fundamental life cycle of *T. lewisi*. At the crisis, it is apparently impossible to force a further number increase from the trypanosomes; nor does the peak number of the infection rise higher in the treated animals than in those which remained untreated. A possible explanation for these facts will be discussed below.

Mechanism of the Increase

. Three possibilities present themselves to account for the observed increases in trypanosomes:

1. The ammonium chloride acts upon the inner organs, contracts their capillaries, and forces the trypanosomes into the peripheral circulation. The increase under these conditions would be apparent only, and would not represent a real increase in the total number of organisms in the body. While ammonium chloride is not known to have such an effect upon the smooth musculature, it was thought worth while to test the possibility by actual experiment. The following experiments were therefore carried out. (a) Ammonium chloride was injected into normal rats. If the solution acts in the assumed way upon the capillaries (*e.g.* of the spleen), the peripheral erythrocyte count should be increased. Ammonium chloride, however, either had no effect upon the peripheral concentration of erythrocytes, or actually caused a slight decrease. For example, the injection of a total of 1.5 cc. during $1\frac{1}{2}$ hours was followed by a decrease in the red blood cell count from 5,940,000 to 5,510,000 in $1\frac{1}{2}$ hours and to 5,660,000 in 2 hours. Pilocarpine given to a normal rat increased the red cell count from 6,740,000 to 6,910,000 in 40 minutes and to 7,380,000 in $1\frac{1}{2}$ hours. (b) If numbers competent to cause the observed increase after ammonium chloride injections are being held in the inner capillaries, the injection of a substance known to contract these capillaries should bring about increases in numbers in the peripheral circulation. Accordingly, pilocarpine was injected into an infected

rat. Altogether, 3.5 mg. were given, and the resulting increase in trypanosomes, from 88,000 to 98,000 (11 per cent) in $4\frac{3}{4}$ hours, was of the same order as that found in uninfluenced infections.

It is evident from these experiments that the injection of ammonium chloride does not draw the trypanosomes from some inner reservoir into the peripheral circulation.

2. The second possibility is that the increase results from increased reproduction of the trypanosomes. Taliaferro and Taliaferro (6) have demonstrated that the rate of reproduction of *T. lewisi* may be determined by finding the variation in length of the trypanosomes. When reproduction is proceeding rapidly, this variation will be greater than when reproduction is infrequent. The size variability within a given sample is expressed by the coefficient of variation (C.V.). The details of the method may be found in their paper. If the coefficient of variation is significantly greater after ammonium chloride injections we would be justified in concluding that the rate of reproduction had been increased.

The results of this study, which was made on nine rats, are given in Table IV. Five of the animals (2, 4, 5, 7, and 9) showed larger coefficients of variation at the end of the experiment than at the beginning; two (3 and 8) showed an increase followed by a decrease; while the remaining two (1 and 6) had decreases in this figure. From the data on each animal, the standard deviation of the coefficient of variation was calculated, and also the standard deviation of the difference between each two successive determinations (σ_{x-y}). This difference is expressed in the last column. From inspection of these figures it is evident that in all cases save two the standard deviation of the difference is equal to or greater than the difference itself. In the two cases where the difference is greater than the standard deviation of the difference, the difference itself represents a fall in the coefficients of variation. These two cases are Rat 3 (4.2 ± 1.81) and Rat 8 (3.5 ± 0.92).

It seems probable from these figures that the differences between the coefficients of variation are due to sampling and technique, and are not the result of actual variations in the reproductive rate of the trypanosomes. One may conclude further that the injection of ammonium chloride does not affect the reproduction of *T. lewisi*.

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TABLE IV

Rate of Reproduction of Trypanosoma lewisi in Rats, before and after Injections of Ammonium Chloride

Rat No.	Variation. Thou- sands per c.mm.	Percent- age increase	Total amount NH ₄ Cl	Time	Stand- ard deviation	Mean length	Coeffi- cient of variation (C.V.)	Stand- ard deviation of C.V.	σ_{x-y}	Difference
				hrs.		microns				
1	25 to 33	32	0.75	1	6.45	22.0	29.0	2.96		
2	20 to 53	165	0.75	4	5.5	23.4	23.5	2.37	3.66	5.5 \pm 3.66
					5.3	24.6	21.5	2.5		
3	11 to 18 to 53	63	0.75	1	6.3	26.0	24.2	2.42	3.48	2.7 \pm 3.48
					5.2	22.5	23.4	1.64		
		194	0.75	3½	5.27	22.09	24.0	1.24	2.05	0.6 \pm 2.05
4	14 to 23 to 43	70	0.75	1½	4.9	24.7	19.8	1.4	1.84	4.2 \pm 1.84
		87	0.75	3	5.76	22.2	25.9	2.6		
					5.14	21.9	23.4	2.36	3.51	2.5 \pm 3.51
5	40 to 56 to 114	40	0.75	3	5.5	24.1	22.8	2.4	3.36	0.6 \pm 3.36
					5.6	22.6	24.7	1.7		
		103	0.75	3	7.0	23.3	30.0	3.0	3.44	5.3 \pm 3.44
6*	65 to 67 to 68 to 73	3	0.125	45 min.	8.86	27.9	31.3	3.1	4.3	1.3 \pm 4.3
					7.48	31.4	23.8	1.68		
		1.4	0.125	30 hrs.	7.1	29.6	23.9	1.68	2.36	0.1 \pm 2.36
					6.72	28.2	23.4	1.63	2.34	0.5 \pm 2.34
		7	0.125	1	6.85	28.7	23.8	1.69	2.34	0.4 \pm 2.34

* Smaller doses of ammonium chloride were given to this animal than to the others.

TABLE IV—*Concluded*

Rat No.	Variation. Thousands per c.mm.	Percent-age increase	Total amount NH_4Cl	Time	Standard deviation	Mean length	Coefficient of variation (C.V.)	Standard deviation of C.V.	σ_{x-y}	Difference
				<i>min.</i>		<i>microns</i>				
7	83				7.47	29.9	24.9	1.77		
	to 92	11	0.5	40	8.63	29.9	28.8	2.05	2.7	3.9 ± 2.7
	to 96	4	0.5	30	7.78	28.2	27.5	1.9	2.78	1.3 ± 2.78
	to 102	6	0.5	1	6.96	27.8	25.0	1.7	2.54	2.5 ± 2.54
	to 109	7	0.0	1	7.31	31.7	23.0	1.6	2.33	2.0 ± 2.33
8	187				2.16	29.5	7.3	0.73		
	to 192	1.5	0.75	6	2.6	31.2	8.2	0.8	1.08	0.9 ± 1.08
	to 201	4.6	0.75	3	1.34	28.0	4.7	0.46	0.92	3.5 ± 0.92
9	201				2.05	29.1	7.0	0.71		
	to 204	1.5	0.75	1½	2.28	28.1	8.1	0.81	1.06	1.1 ± 1.06
	to 215	5	0.75	3	2.57	30.7	8.3	0.84	1.16	0.2 ± 1.16

3. As the Taliaferros have stated, the number of parasites in the blood at a given time equals those produced by reproduction less those destroyed by the host's defense mechanism. Since the possibility of increased reproduction has been eliminated, and since ammonium chloride itself does not increase the peripheral concentration of trypanosomes by contracting the capillaries of the inner organs, it is evident that the observed increases must result from a lessening of the number of trypanosomes being destroyed. Although the evidence for this statement is indirect, it is difficult to see from what other source the increase could come than from the inhibiting effect of the hydrochloric acid upon the destructive mechanism, whether this be cellular or humoral.

DISCUSSION

The increased numbers of trypanosomes in the blood, under the conditions of these experiments, probably represent the cellular reaction

a normal rate of reproduction plus the unusual number which have escaped destruction. That the observed increases are large in the early stages of the disease and small or absent during and after the crisis finds its explanation, under the same hypothesis, in the known fact that the destructive mechanism for *T. lewisi* is at a minimum early in the infection, and is therefore easily inhibited by ammonium chloride. As already pointed out, we have not been able to influence fundamentally the normal life cycle of this parasite, an observation explainable on the basis that the destructive mechanism becomes more active and efficient as the disease progresses, so that the injection of even large amounts of ammonium chloride over relatively long periods of time is without effect.

Kligler, Geiger, and Comaroff (1) have performed experiments which are, in a way, the reverse of those reported here. They attempted to mitigate the acidosis and to restore the alkali reserve to normal levels in infected rats by the injection of sodium bicarbonate throughout the course of the disease. The length of life of animals so treated was nearly 50 per cent greater than in the uninfluenced controls. Since they did not report actual counts of trypanosomes, it is impossible to know whether the rate of increase was slower in the bicarbonate-injected animals than in the controls. Their results are highly suggestive, however, in view of the work reported here.

We have similarly attempted, with our strain of *T. equiperdum*, to increase the length of life of infected animals. In this undertaking we were unsuccessful in that the treated rats, which were given injections of 0.5 cc. of 4 per cent NaHCO_3 at 2 hour intervals, died at about the same time as the control animals, that is, in from 48 to 72 hours. The virulence of our strain, as is evident, is extremely high, and correspondingly difficult to influence, while the *T. evansi* used by Kligler and his coworkers caused a much more extended disease, which lasted, in the experiments cited, for 18 days on the average in the control rats. The difference in our results may find its explanation in this fact.

Andrews and his collaborators (3), who used a strain of *T. equiperdum* only slightly less virulent than ours, were also unable to extend the life of infected rats by means of injections of sodium bicarbonate. It is suggested that the pathogenicity of *T. equiperdum*, in its early

growth stage in the rat, is intimately bound up with an enzymatic peculiarity of its metabolism which leads it to form organic acids from carbohydrates. The accumulation of these acids depresses the destructive mechanism in the host, as we have shown to happen in artificial acidosis in *T. lewisi* infections. The suppression of defense is practically complete. In the case of infections of this type, Taliaferro and Taliaferro (6) have shown that there is no appreciable parasitocidal action. With the accumulation of acid, and the consequent absence of defense on the host's part, a basis is laid for the events which follow—anorexia and death from asphyxia. It seems possible, from the work of Andrews and his collaborators, that these events include an agglutination of the trypanosomes. The agglutination which they describe as occurring "for reasons unknown at present," should be investigated further for a possible relationship to "acid agglutination." In any event, we believe that the suppression of destruction of the trypanosomes begins at a much earlier stage in the infection and does not therefore exclude the possibility of a later spontaneous agglutination.

From the biochemical point of view, *T. lewisi* may be supposed to have the enzymatic peculiarity of breaking carbohydrates completely. Its host consequently suffers no metabolic derangement, and the organism is therefore not pathogenic, in so far as pathogenicity means power to injure the host. In the study cited above (4), Linton showed that the factors which varied in infections with *T. equiperdum*, i.e. lipid phosphorus, lecithin, CO₂-combining capacity, and liver glycogen, were unchanged in *T. lewisi* infections; nor was the blood sugar concentration changed by this organism (7).

Trypanosoma lewisi infections cannot, of course, be considered purely from a biochemical standpoint, since the host-parasite relations have biological peculiarities such as the production of trypanolytic antibodies, and the reproduction inhibiting substance of Taliaferro. In spite of this complication, however, it still serves as a useful biochemical contrast in the study of the pathogenic trypanosomes.

SUMMARY AND CONCLUSIONS

When the alkali reserve is artificially lowered in rats infected with *Trypanosoma lewisi*, the number of parasites in the blood is increased.

The increase is large in the early stages of the disease, and becomes less marked as the number crisis is approached. Near the crisis, and after it, a lowered alkali reserve does not affect the number of trypanosomes.

It has been shown that the observed increase does not result from a contraction of the capillaries of the inner organs, which would throw a large number of trypanosomes into the peripheral circulation; nor is the increase due to a greater reproductive activity on the part of the trypanosomes. The increase must, therefore, be due to an inhibition of the destructive forces of the host.

It is suggested that the known production of organic acids by the pathogenic trypanosomes plays a similar rôle in inhibiting the destructive mechanism of the host, and is therefore of significance in the pathogenic activity of these organisms.

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IN VITRO TRANSFORMATION OF PNEUMOCOCCAL TYPES

I. A TECHNIQUE FOR INDUCING TRANSFORMATION OF PNEUMOCOCCAL TYPES IN VITRO

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Griffith (1) was the first to show that S forms of pneumococci could be transformed from one specific S type into other specific S types through the intermediate stage of the R form. In recent publications by one of the authors (2, 3), Griffith's observations on the transformation of pneumococcal types were confirmed and extended. The results of these investigations may be summarized as follows:

It was shown that R forms of pneumococci could be converted into S forms of the original type by the subcutaneous injection, in white mice, of small amounts of living R organisms, together with S vaccines of the homologous type. (In this procedure, vaccines prepared from cultures of Type II S and Type III S organisms are equally effective whether heated for 15 minutes at 60°, for 15 minutes at 80° or for 15 minutes at 100°C. Vaccines prepared from Type I S organisms, however, are effective in producing reversion when heated for 15 minutes at 60°C. and for 15 minutes at 80°C., but not when heated for 15 minutes at 100°C.) *In vitro* attempts to effect the R → S reversion by the use of vaccines were uniformly unsuccessful.

It was further shown that R forms of pneumococci, derived from S forms of any specific type, could be transformed into S organisms of other specific types by the following procedure:—The subcutaneous injection, in white mice, of small amounts of living R forms together with vaccines of heterologous S cultures. (The following points were established:—(a) S vaccines, heated for 15 minutes at temperatures between 60° and 80°C., are effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine. (b) S vaccines heated for 15 minutes at temperatures between 80° and 100°C. are not effective

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in causing such transformations. (c) S vaccines heated for periods as long as 2 hours at 60°C. are effective in inducing transformation of type.) All attempts to induce transformation of type by *in vitro* methods were unsuccessful.

The studies recorded in the present communication are concerned with successful experiments on transformation of pneumococcal types by *in vitro* procedures. Previous unsuccessful attempts to effect similar transformation of type by *in vitro* procedures may be summarized as follows:

R pneumococci were cultured in blood broth to which were added the bacteria from 100 cc. of heterologous S cultures, killed by heating for 15 minutes at 60°. Transfers were continued for fifteen subcultures in this medium without the appearance of S colonies. The addition of anti-R serum (4, 5) to culture media containing S vaccines likewise failed to induce transformation. In other experiments, R forms were grown, under vaseline seal, in blood broth to which was added lymph tissue, muscle tissue and ground-up spleen, as well as large amounts of S vaccines. All such *in vitro* attempts to effect transformation of R forms into S forms of heterologous types yielded negative results. In a further attempt to secure transformation of type by *in vitro* methods the following experiment was done. A series of mice was injected intraperitoneally with large amounts of S vaccine. The animals were sacrificed at varying intervals and the peritoneal contents washed out with sterile saline. The washings were added to plain broth and inoculated with R forms. In no instance was transformation of type effected by this procedure.

In seeking an explanation of the failure to induce transformation of type by the *in vitro* methods previously adopted, two possibilities presented themselves:—either the conditions employed were unsuitable or living tissues were necessary for the transformation process. Before resorting to the latter explanation further *in vitro* studies were undertaken.

Methods

The suspensions of heat-killed organisms were prepared in the same manner as described in previous communications (2, 3). Similar control measures were adopted to establish beyond question the sterility of the vaccines:

1. Cultures were made from all vaccines in blood broth and on blood agar plates. In many experiments this was done in varying dilutions. In no instance was growth obtained.

2. In all experiments mice were injected with large amounts of S vaccine alone. Without exception all such animals survived.

Other controls will be detailed in the description of certain experiments. In

the course of the present and previous investigations pneumococcal vaccines have been employed in several hundred carefully controlled experiments. In no instance has it been possible to demonstrate the persistence of viable bacteria in the vaccines employed.

The R cultures of pneumococci employed were obtained in the usual way by growing S organisms in homologous immune serum. The nature of the R cultures was frequently proven during the course of the experiments in the following manner:—Suitable amounts of the R cultures were injected subcutaneously in white mice. Under these conditions (1, 2) the R forms invariably reverted to the S form of the type from which they had been originally derived.

The term "anti-R" serum, as employed in the present communication, requires some explanation. Avery and Heidelberger (6) showed that type-specific, anti-pneumococcus sera contain not only type-specific (anti-S) antibodies, but also antibodies reacting with the protein substance, which is common to all pneumococci. Reimann (7) subsequently showed that sera prepared with R forms are immunologically similar to sera prepared with the protein of pneumococcus. In the experiments to be reported anti-S serum of heterologous type was therefore used as a convenient source of anti-R antibodies.

The following procedure was adopted in identifying the organisms obtained in transformation experiments.

The cultures were streaked on blood agar plates which were allowed to incubate at 37°C. for 18 to 24 hours. At the end of this period the colonies were identified under a Zeiss colony microscope. Frequently it was found convenient to allow the plates to remain at room temperature for a further period of 24 hours. In no instance, however, was colony morphology alone used as the sole criterion of the nature of the organisms composing the colonies. In all transformation experiments the results were confirmed by agglutination tests carried out on cultures obtained from individual colonies.

EXPERIMENTAL

As a preliminary step *in vitro* experiments were arranged in an attempt to effect reversion of R forms to S forms of the original type by the use of homologous S vaccines.

A series of mice was injected intraperitoneally with III S vaccine. The animals were killed after intervals of 2, 4 and 6 hours. The peritoneal contents were washed out with sterile saline and the recovered material was seeded with a 3 R culture. The technique adopted varied in two particulars from that which had previously been employed: (1) very small seedings, representing 1 drop of a 10^{-4} dilution of the R culture, were used; (2) the cultures were allowed to incubate for several days at 37°C. and blood agar plates were streaked at 24 hour

Experiments to Determine the Effect of Seeding Varying Amounts of an R Culture in Media Containing Heterologous S Vaccine

It has been pointed out that the technique employed in the foregoing experiments varied in certain particulars from that which had been adopted in previous unsuccessful *in vitro* experiments. One of these variations consisted in the use of very small seedings of the R culture. It was therefore of interest to determine the effect of inoculating media containing S vaccine with various amounts of an R culture. Media containing samples of III S vaccine were seeded with 1 drop of varying dilutions of a 2 R culture. The details of the experiment appear in Table I.

TABLE I

The Effect of Inoculating Media Containing S Vaccines with Varying Amounts of Living R Forms Derived from Heterologous S Types

2 R culture, 1 drop in dilutions as detailed; 0.5 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine, equivalent of 20 cc. of original culture.

Dilutions of 2 R culture	Colonies on plates streaked		
	24 hours	48 hours	72 hours
Whole culture	R only	R only	R only
10 ⁻¹ dilution	" "	" "	" "
10 ⁻² "	" "	Several III S	Nearly all III S
10 ⁻³ "	" "	Numerous " "	" " " "
10 ⁻⁴ "	" "	" " "	" " " "
10 ⁻⁵ "	" "	" " "	" " " "
10 ⁻⁶ "	" "	" " "	" " " "

Controls: III S vaccine, 1 loopful and 1 drop, cultured in blood broth and on blood agar plates:—No growth in 72 hours.

Two mice, injected intraperitoneally with III S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 10 days.

The results presented in Table I show that transformation of type as obtained only when relatively small seedings of the R culture were used. The experiment was repeated and similar results were obtained.

On this occasion numerous III S colonies were again recovered in all instances which the seeding of the R culture was less than 1 drop of a 10⁻¹ dilution. One III S colony was recovered after 24 hours from the 10⁻¹ seeding; likewise

one III S colony was recovered after 96 hours from the whole culture seeding. All the remaining colonies obtained from the whole culture seeding and the 10^{-1} dilution seeding were of the R variety.

These experiments demonstrated the importance of employing small amounts of the R culture and afforded an explanation for the failure of many previous attempts to secure transformation of type by *in vitro* methods.

Experiments to Determine the Amount of an S Vaccine Necessary to Effect Transformation of Type in Vitro

Both for theoretical and practical reasons it was of importance to determine the amount of S vaccine necessary to effect transformation by the *in vitro* procedure.

Six sterile agglutination tubes were set up, in duplicate, containing varying dilutions of a III S vaccine, amounts representing the bacteria from 100, 50, 25, 10, 5 and 1 cc. of the original culture. To each tube were added 0.5 cc. blood broth and 0.1 cc. anti-R serum. Six of the tubes were inoculated with 1 drop of a 10^{-6} dilution of a 2 R culture. The remaining six were kept as controls. For further control purposes two mice were injected intraperitoneally with 0.5 cc. of the vaccine, representing 100 cc. of culture. The control mice were alive and well when sacrificed after a period of 10 days. All the tubes were incubated at 37°C . and blood agar plates were streaked at 24 hour intervals for 5 days. The results of the experiment appear in Table II.

The data presented in Table II show that transformation of type can be effected *in vitro* by the use of small amounts of S vaccine, amounts representing the bacteria from as little as 1 cc. of the original cultures. This experiment was repeated many times and similar results were obtained. The smallest amount of vaccine proving effective in any experiment was that representing the bacteria from 0.1 cc. of the original culture.

Determination of Culture Media Necessary to Effect Transformation of Type in Vitro

In the experiments carried out up to this point blood broth and anti-R serum were added to the broth suspensions of S vaccines as culture media for the growth of R forms. Experiments were next

arranged to determine whether the presence of these additional substances was necessary for the transformation process.

The following variations in culture media were employed: plain broth alone, plain broth and normal rabbit serum, plain broth and anti-R serum; blood broth alone, blood broth and normal rabbit serum, blood broth and anti-R serum. The results of the experiment appear in Table III.

TABLE II

The Amount of Heat-Killed Suspensions of S Forms Necessary to Induce Transformation of Type in Vitro

2 R culture, 1 drop 10^{-6} dilution; 0.5 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine, amounts as detailed.

III S vaccine (in cc. of original culture)	Colonies on plates streaked	
	24 hours	48 hours
100	Nearly one-half III S	Nearly all III S
50	" " " "	" " " "
25	Numerous " "	" " " "
10	20-30 " "	About one-half " "
5	6-10 " "	" " " "
1	A few " "	Numerous " "
Controls: 0.5 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine		
100	No growth	No growth
50	" "	" "
25	" "	" "
10	" "	" "
5	" "	" "
1	" "	" "

Two mice, injected intraperitoneally with III S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 14 days.

The results presented in Table III show that transformation of type was effected in all instances except those in which plain broth alone was used as the culture medium. Type III S colonies appeared more abundantly in those instances in which both blood broth and serum were employed. Normal rabbit serum, however, appeared to be somewhat less effective than anti-R serum. The experiment was repeated on two occasions and comparable results were obtained. In

subsequent experiments both blood broth and anti-R serum were therefore used as culture media in transformation experiments.

TABLE III

Culture Media Necessary to Induce Transformation of Type in Vitro

2 R culture, 1 drop 10^{-6} dilution; III S vaccine, equivalent of 10 cc. of original culture; culture media, as detailed.

Culture media	Colonies on plates streaked			
	24 hours	48 hours	72 hours	96 hours
0.6 cc. plain broth	1 R only	R only	R only	R only
	2 " "	" "	" "	" "
0.5 cc. plain broth and 0.1 cc. anti-R serum	3 " "	" "	Several III S	Numerous III S
	4 " "	" "	R only	R only
0.5 cc. plain broth and 0.1 cc. normal rabbit serum	5 " "	One III S	A few III S	" "
	6 " "	R only	R only	" "
0.6 cc. blood broth	7 " "	Several III S	Numerous III S	Nearly all III S
	8 " "	R only	R only	R only
0.5 cc. blood broth and 0.1 cc. anti-R serum	9 " "	One III S	Numerous III S	Nearly all III S
	10 " "	Numerous III S	Nearly all III S	" "
0.5 cc. blood broth and 0.1 cc. normal rabbit serum	11 " "	A few III S	Several III S	Numerous III S
	12 " "	R only	R only	R only

Controls: III S vaccine, 1 loopful and 1 drop, cultured in blood broth and on blood agar plates:—No growth in 72 hours.

Two mice, injected intraperitoneally with III S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 10 days.

Thermal Differentiation

It was previously pointed out (3) in the *in vivo* experiments on transformation of type that the temperature at which an S vaccine is heated materially affects the results obtained. S vaccines heated for

15 minutes at 80°C. proved effective in inducing transformation of type. However, S vaccines heated for a like period at 90°C. and 100°C. were ineffective. It was a matter of considerable interest to determine whether similar results would be obtained in the *in vitro* procedure.

The bacteria from 240 cc. of a III S culture were suspended in 1.2 cc. of plain broth and divided into two equal portions of 0.6 cc. each. One sample was heated for 15 minutes at 80°C. and the other for 15 minutes at 100°C. The two lots of vaccine were used in transformation experiments as detailed in Table IV.

TABLE IV

The Effect of the Temperature at Which an S Vaccine Is Heated upon Its Efficacy in Inducing Transformation of Type

2 R culture, 1 drop 10⁻⁶ dilution; 0.5 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine, equivalent of 30 cc. of original culture, heated for 15 minutes at temperatures indicated.

Temperature at which S vaccine was heated		Colonies on plates streaked		
		24 hours	48 hours	72 hours
80	1	Few III S	Several III S	All III S
	2	No " "	Few " "	" " "
100	3	" " "	No " "	No " "
	4	" " "	" " "	" " "

Controls: III S vaccine, 1 loopful and 1 drop, cultured in blood broth and on blood agar plates:—No growth in 72 hours.

Two mice injected intraperitoneally with III S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 10 days.

The data presented in Table IV show that an S vaccine heated for 15 minutes at 80°C. was effective in inducing transformation of type. An S vaccine heated for 15 minutes at 100°C., however, failed to induce the change.

In other experiments the vaccine was heated for a period of 15 minutes at temperatures of 60°, 70°, 80°, 90° and 100°. The results obtained showed slight variations with different lots of vaccine. Generally speaking, however, S vaccines heated for 15 minutes at

temperatures up to and including 80°C. proved effective in inducing transformation of type, while those heated for a similar period at 90° and 100° were uniformly ineffective.

The effect of heating an S vaccine for successive periods at various temperatures was next determined.

Aliquot portions of a III S vaccine were heated for 15 minutes for three successive occasions at temperatures of 60°, 70°, 80°, 90° and 100°C. The results of the experiment appear in Table V.

The results detailed in Table V show that a III S vaccine, heated for 15 minutes on three successive occasions at 60°C., retained its effectiveness in inducing transformation of type. Likewise a III S vaccine heated for 15 minutes on two successive occasions at 70°C. and for one period of 15 minutes at 80° was similarly effective in bringing about the change. However when heated for three successive periods of 15 minutes at 70°, and for two periods of 15 minutes at 80°, the vaccine was apparently rendered ineffective. Vaccines heated for as short a period as 15 minutes at 90° and 100°C. uniformly failed to induce transformation.

Duration of Heating

The following experiment was done to determine the effect of heating on S vaccine for a prolonged period at 60°C.

A suspension of III S organisms was heated at 60°C. At hourly intervals for 4 hours samples of the vaccine were withdrawn and used in transformation experiments. The results appear in Table VI.

The data presented in Table VI indicate that a III S vaccine, heated for 4 hours at 60°C., still retained its effectiveness in inducing transformation of type. The results suggest, however, that the samples of vaccine heated for periods of 3 hours and 4 hours at 60°C. were somewhat less effective than those lots which were heated for periods of 1 hour and 2 hours at the same temperature.

Transformation of a 2 R Culture into Type I S Organisms

In all *in vitro* experiments carried out up to this point a 2 R culture and III S vaccine were employed. In previous *in vitro* experiments

TABLE V
Effect of Heating an S Vaccine for Successive Periods of 15 Minutes at Various Temperatures between 60° and 100°C.

2 R culture, 1 drop 10⁻⁴ dilution; 0.7 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine, equivalent of 20 cc. of original culture, heated as indicated.

Tempera- ture at which vaccine was heated	No. successive 15 minute periods	Colonies on plates streaked				
		24 hours	48 hours	72 hours	96 hours	120 hours
60	One	No III S	No III S	Several III S	Nearly all III S	All III S
		" " "	" " "	Numerous " "	" " "	" " "
	Two	" " "	" " "	No " "	No " "	Several " "
		" " "	" " "	Numerous " "	Numerous " "	Numerous " "
	Three	" " "	" " "	" " "	" " "	" " "
		" " "	" " "	" " "	" " "	" " "
70	One	" " "	" " "	Several " "	" " "	Nearly all " "
		" " "	" " "	Numerous " "	" " "	" " "
	Two	" " "	" " "	No " "	Several " "	Numerous " "
		" " "	" " "	" " "	No " "	" " "
	Three	" " "	" " "	" " "	" " "	No " "
		" " "	" " "	" " "	" " "	" " "
80	One	" " "	" " "	Numerous " "	Nearly all " "	Nearly all " "
		" " "	" " "	No " "	Several " "	Numerous " "
	Two	" " "	" " "	" " "	No " "	No " "
		" " "	" " "	" " "	" " "	" " "
	Three	" " "	" " "	" " "	" " "	" " "
		" " "	" " "	" " "	" " "	" " "

[illegible]

Controls: III S vaccine, heated for 15 minutes at 60°C., 1 loopful and 1 drop, cultured in blood broth and on blood agar plates: -No growth in 72 hours.

Two mice injected intraperitoneally with III S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 10 days.

(3) it had been demonstrated that an R culture, derived from S forms of any type, could be transformed into S forms of any other type by the use of S vaccines of the appropriate type. It therefore seemed of interest to determine whether the 2 R culture that had been transformed into III S organisms by the *in vitro* procedure could be similarly transformed into I S organisms by the use of a Type I S vaccine.

TABLE VI

The Effect of the Duration of Heating upon the Efficacy of an S Vaccine in Inducing Transformation of Type

2 R culture, 1 drop 10^{-6} dilution; 0.5 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine, equivalent of 10 cc. of original culture, heated at 60° for periods indicated.

Duration of heating at 60°C.	Colonies on plates streaked			
	24 hours	48 hours	72 hours	96 hours
hrs.				
1 {1	R only	Numerous III S	Over half III S	Nearly all III S
2 {2	" "	" " "	" " " "	" " " "
2 {3	" "	" " "	" " " "	" " " "
3 {4	" "	" " "	" " " "	" " " "
3 {5	" "	Suggestive III S only	Suggestive III S only	Suggestive III S only
4 {6	" "	One III S	A few III S	Several III S
4 {7	" "	A few " "	Several " "	" " "
8	" "	Suggestive III S only	Suggestive III S only	Suggestive III S only

Controls: III S vaccine, 1 loopful and 1 drop cultured in blood broth and on blood agar plates:—No growth in 72 hours.

Two mice injected intraperitoneally with III S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 10 days.

A Type I S vaccine was prepared in the usual way from a virulent Type I S culture. A sample of the I S vaccine was seeded with a 2 R culture under the conditions outlined in Table VII.

Considerable difficulty was encountered in identifying the colonies which appeared on plates streaked from the cultures after 48 hours incubation. Several apparently smooth colonies were picked from the plates and transferred to blood broth. Agglutination tests on the

resulting cultures gave a typical Type I agglutination in all instances. The virulence of these cultures was determined by mouse inoculation. They were found to be of maximal virulence and cultures from the hearts' blood of the infected animals gave a typical Type I S agglutination. It therefore appeared that a 2 R culture could be transformed with equal ease into III S organisms or into I S organisms, according to the type of S vaccine employed.

Further experiments on transformation of pneumococcal types by *in vitro* procedures are described in the following paper.

TABLE VII

Transformation of R Forms Derived from a II S Culture into S Forms of Type I
2 R culture, 1 drop 10^{-6} dilution; 0.5 cc. blood broth; 0.1 cc. anti-R serum;
I S vaccine, equivalent of 40 cc. of original culture.

	Colonies on plates streaked			
	24 hours	48 hours	72 hours	96 hours
1	R only	R only ?	Several I S ?	Several I S ?
2	" "	" " ?	" " " ?	" " " ?

Several suggestive I S colonies were picked and cultured in blood broth. Resulting growth gave Type I agglutination in all instances.

Controls: I S vaccine, 1 loopful and 1 drop, cultured in blood broth and on blood agar plates:—No growth in 72 hours.

Two mice injected intraperitoneally with I S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 10 days.

DISCUSSION

The discussion of the results presented in the foregoing communication will be confined to a consideration of the conditions under which transformation of pneumococcal types may be effected by *in vitro* procedures.

In previous studies (2, 3) it had been shown that S pneumococci could be transformed from one specific S type into other specific S types through the intermediate stage of the R form. In this transformation process *in vitro* procedures were employed. In the present communication it has been demonstrated that similar transformations of type may be effected entirely by *in vitro* methods. The *in vitro* method

consists in growing small amounts of an R culture in suitable culture media to which has been added a vaccine of heterologous S type. Under these conditions the R forms may be transformed into S forms of the same type as the vaccine.

In considering this phenomenon the first possibility which suggests itself is that the transformation may be apparent rather than real, and may be due to the survival of S organisms in the vaccines employed. This possibility has received full consideration in previous communications (2, 3) on transformation of type by *in vivo* procedures. In the course of the present and previous investigations pneumococcal vaccines have been employed in several hundred carefully controlled experiments. In no instance has it been possible to demonstrate the occurrence of viable organisms in the vaccines employed. The conclusion must therefore be drawn that, if the transformation is due to the persistence of living bacteria in the vaccines, the surviving cells do not conform to any recognized form of bacterial life.

The suggestion that the transformation may only be the result of a temporary acquisition, by the R form, of the attributes of the S cell, has also received full consideration in previous communications. It was shown (3) that the newly formed S organisms not only possessed all the characteristics of type-specific S cells but that they continued, when subcultured, to reproduce S forms apparently indefinitely. The results of the experiments therefore indicate that, under the conditions provided, the R cell acquires the capacity of elaborating a specific polysaccharide of the same type as that of the vaccine in which it has been grown.

The conditions under which transformation of type may be effected merit some consideration. It was shown that relatively small inocula of R cells provide the most suitable conditions. Large inocula usually resulted in the growth of R forms only. The growth of large numbers of R pneumococci apparently created conditions unfavorable for transformation. No adequate explanation of this finding is offered. The suggestion is made, however, that the unsuitable conditions may have been produced by the elaboration of bacterial peroxide by the large amounts of R inocula. Further experiments are required to establish this point.

In the experiments reported very small quantities of vaccine, quantities representing the bacteria from as little as 0.1 cc. of the original culture, were effective in inducing transformation of type. Smaller quantities of vaccine, representing the bacteria from less than 0.1 cc. of the original culture, proved ineffective. Attempts to extract the essential factor responsible for transformation are described in the following paper.

In effecting transformation of type by *in vitro* procedures the nature of the culture media employed appears to be a matter of considerable importance. In all successful experiments either serum or red blood cells were added to the culture media. When plain broth was used transformation failed to occur. The suggestion is offered that the addition of the blood and serum may have afforded a convenient source of catalase and peroxidase. In the absence of these substances sufficiently reduced conditions may not have been present. Further experiments relating to this point are described in the following paper.

In previous communications Dawson and Avery (4) and Dawson (5) showed that the growth of R pneumococci in media containing anti-R serum frequently resulted in the conversion of R forms into S forms of the original type. For this reason anti-R serum was employed in the majority of the transformation experiments reported in this study. During the course of the investigation it was found that, although the use of anti-R serum appeared to facilitate transformation, the change could frequently be effected in the absence of anti-R antibodies.

The observation that the property of an S vaccine responsible for transformation of type is thermolabile is of considerable interest. Transformation was effected with S vaccines which had been heated for a period of 4 hours at 60°C. The change was also induced with vaccines heated for a period of 15 minutes at 70° and for 15 minutes at 80°C. However, vaccines heated for a similar period at 90° and at 100°C. were not effective. Furthermore, transformation was induced with vaccines heated for 15 minutes on three successive occasions at 70°C., for the same period on two successive occasions at 70°C. and for one period of 15 minutes at 80°C. Vaccines heated for a period of 15 minutes on three successive occasions at 70°C. and for a similar period on two successive occasions at 90°C. were not effective. Refer-

ence will be made to the significance of these observations in the following paper.

In vitro experiments have been described in which R forms of pneumococcus, derived from a Type II S culture, have been transformed into S forms of Type I and into S forms of Type III, according to the type of vaccine employed. This observation supports previously described *in vivo* transformation experiments in which it was shown that R forms, derived from one specific type, may be transformed into S forms of any other specific type (3).

The nature of the mechanism by which transformation of type may be brought about by *in vitro* procedures and the significance of these findings in the field of bacteriology and epidemiology will be discussed in the accompanying paper.

SUMMARY AND CONCLUSIONS

1. Type-specific S pneumococci may be transformed from one specific S type into other specific S types entirely by *in vitro* methods.
2. R forms of pneumococci, derived from S forms of one specific type, may be transformed into S forms of other specific types by the following *in vitro* procedure:—the growth of small inocula of R forms in media containing vaccines prepared from heterologous S cultures.
3. Transformation of type may be effected in this procedure by the use of small quantities of S vaccine,—quantities representing the bacteria from as little as 0.1 cc. of the original culture.
4. Transformation of type, as induced by this procedure, is most readily effected by employing anti-R serum in the culture medium. Transformation of type may be effected, however, in media which do not contain anti-R antibodies.
5. Previous findings on the thermal characteristics of the property of S vaccines responsible for transformation of type have been confirmed and extended.

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IN VITRO TRANSFORMATION OF PNEUMOCOCCAL TYPES

II. THE NATURE OF THE FACTOR RESPONSIBLE FOR THE TRANSFORMATION OF PNEUMOCOCCAL TYPES

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In the accompanying communication (1) a method of inducing transformation of pneumococcal types entirely by *in vitro* procedures is reported. In the present study the conditions under which transformation of pneumococcal types can be effected by *in vitro* procedures have been further analyzed and the factor responsible for transformation has been investigated.

The Nature of R Cultures Most Suitable for Transformation of Type by in Vitro Methods

As originally pointed out by Griffith (2), and subsequently confirmed by one of the authors (3), there are varying degrees of constancy or stability of the R variant of pneumococcus. In previous work on transformation of type by *in vitro* procedures it was found that the nature of the R culture employed materially affected the results (4, 5).

Thus, in the *in vitro* procedure, it was shown that an R culture which possessed only a slight degree of R stability showed a tendency to revert to the S form of the original type. However, R cultures which were more definitely stabilized in the R form showed less tendency to revert to the S form of the original type and were more readily transformed into S forms of heterologous types. R cultures which were still more definitely stabilized in the R form were more "resistant" and could be converted into S forms of either homologous or heterologous types only with considerable difficulty.

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In the *in vitro* experiments R cultures of varying degrees of stability were employed. The degree of R stability was determined by one of the following methods: (1) the number of transfers necessary to effect reversion in 10 per cent anti-R serum broth (3, 6); (2) the amount of living R culture necessary to induce reversion when injected subcutaneously in white mice; (3) the ease with which transformation of type could be effected by *in vivo* procedures.

The results obtained in *in vitro* experiments in which various R cultures were employed may be presented in brief as follows: (1) transformation of type was most readily effected with those R cultures which possessed only slight degrees of R stability; (2) transformation of type was not effected with certain cultures which were more definitely stabilized in the R form; (3) under the conditions employed, the R cultures, in the presence of heterologous S vaccines, showed little tendency to revert to S forms of the original type.

The significance of these observations will be referred to in a subsequent communication.

TABLE I

The Effect of Growing S Organisms in Media Containing R Vaccine

S culture, 1 drop 10⁻⁶ dilution; 0.5 cc. blood broth; 0.1 cc. normal rabbit serum; 1 R vaccine, equivalent of 100 cc. of original culture.

Type of culture used for inoculation	Colonies on plates streaked			
	24 hours	48 hours	72 hours	96 hours
I S	I S only	I S only	I S only	I S only
" "	" " "	" " "	" " "	" " "
II "	II " "	II " "	II " "	II " "
" "	" " "	" " "	" " "	" " "
III "	III " "	III " "	III " "	III " "
" "	" " "	" " "	" " "	" " "

*Attempts to Convert S Forms into R Forms by Growth in Media
Containing R Vaccines*

As detailed in the preceding experiments, the growth of R forms of pneumococci, under suitable conditions, in media containing S vaccines of heterologous type, frequently resulted in the transformation of the R forms into S forms of the type of the vaccine. The effect of growing S organisms in media containing R vaccines was next determined.

Type I S, Type II S and Type III S organisms were grown in media to which were added relatively large amounts of a 1 R vaccine. Very small seedings of the S cultures were employed and plates were streaked from the cultures every 24 hours for 4 days. Normal rabbit serum was used in the culture media instead of anti-R serum. The results of the experiment appear in Table I.

From the data presented in Table I it appears that S organisms are capable of living and multiplying in the presence of large amounts of R vaccine. Under the conditions employed the S forms showed no tendency to revert to the R form. No attempt was made to determine the effect of serial transplants of S organisms in media containing R vaccines.

Attempts to Effect Transformation of Type by the Use of (1) the Supernatant Fluid from an S Vaccine, (2) the Filtrate of an S Vaccine, (3) Purified Specific Soluble Substance

In previous experiments it was found that the smallest amount of an S vaccine effective in inducing transformation of type was that representing the bacteria from 0.1 cc. of the original S culture. It therefore appeared improbable that either the supernatant material or the filtrate of an S vaccine would prove effective in inducing the change. However, in order to analyze further the conditions responsible for transformation, experiments were done using the supernatant and the filtrate obtained from an S vaccine.

A III S vaccine was prepared in the usual way and divided into two equal lots. One lot was centrifuged at high speed for 1 hour. At the end of this time the supernatant material was removed and used in transformation experiments. The other lot of vaccine was filtered through a Berkefeld candle and the filtrate similarly employed in transformation experiments. Relatively large amounts of the supernatant material and the filtrate were employed. Transformation of type was not obtained in any instance.

The experiment was repeated many times with many variations in technique but the results were uniformly negative.

In other experiments attempts were made to effect transformation of type by the use of purified specific soluble substance of Type III S pneumococcus¹ (7). All such experiments were unsuccessful.

¹We are indebted to Dr. M. Heidelberger of the College of Physicians and Surgeons, Columbia University, for placing material at our disposal for these experiments.

Further experiments in which the filtrate of an actively growing S culture was employed also yielded negative results.

Attempts to Effect Transformation of Type by Extracts of S Organisms

In all successful transformation experiments carried out up to this point a suspension of S cells, killed by heating, was employed. Experiments were next devised in an attempt to effect transformation by the use of cell-free extracts of S organisms. A convenient method of preparing such extracts appeared to be by the method of freezing and thawing. The following experiment was accordingly arranged.

The bacteria obtained by centrifugation from 1200 cc. of a III S culture were taken up in 12 cc. of plain broth and divided into two equal lots of 6 cc. each, designated as Lots A and B. Lot A was heated for 30 minutes at 60°C. At the end of this period 1.5 cc. was withdrawn for experimental and control purposes. The remainder, 4.5 cc., was frozen and thawed twenty-three times in an attempt to break up the cells. It was found, however, that bacterial cells which had previously been subjected to heating for 30 minutes at 60° were very resistant to disruption by this method. The resulting material still showed an abundance of well formed cells when stained by Gram. 0.5 cc. of the heated, frozen and thawed material was withdrawn for experimental purposes. The remaining 4.0 cc. of this material was centrifuged and the supernatant filtered through a Berkefeld candle. The resulting filtrate was used in transformation experiments. Lot B, which had not been subjected to heating, was frozen and thawed twenty-three times. At the end of this time, smears showed only Gram-negative detritus and no intact cells. 1.5 cc. of this material was withdrawn and heated for 30 minutes at 60°C. This material was used for control and experimental purposes. The remainder, 4.5 cc., of the frozen and thawed material was centrifuged and filtered through a Berkefeld candle. The resulting filtrate was employed in transformation experiments. The remaining details and results of this experiment appear in Table II.

The data presented in Table II show that a III S vaccine, heated for 30 minutes at 60°C., was effective in inducing transformation of type. The same vaccine, when subjected to the procedure of freezing and thawing twenty-three times, was likewise effective in inducing transformation. It is again emphasized, however, that the freezing and thawing procedure failed to break up the previously heated cells. The filtrate from the heated, frozen and thawed material was not effective in inducing transformation. Likewise the material which was first frozen and thawed, and subsequently heated at 60°C., failed

to induce transformation of type. Finally, the filtrate of this material was also ineffective. These results may be set down in tabular form as follows:

Suspension of S Cells

1. Heated 30 min. at 60°C.....Effective
2. Heated 30 min. at 60°C., frozen and thawed.....Effective
3. Heated 30 min. at 60°C., frozen and thawed, filtered.....Not effective
4. Frozen and thawed, heated 30 min. at 60°C.....Not effective
5. Frozen and thawed, heated 30 min. at 60°C., filtered.....Not effective

TABLE II

Attempts to Effect Transformation of Type by the Use of Cell-Free Extracts of S Organisms

2 R culture, 1 drop 10^{-8} dilution; 0.5 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine and vaccine "extracts" as indicated.

Method of preparing vaccines and vaccine "extracts"	Colonies on plates streaked			
	24 hours	48 hours	72 hours	96 hours
Heated 30 min. 60°	1 Numerous III S	Nearly all III S	Nearly all III S	Nearly all III S
	2 " "	" "	" "	" "
Heated 30 min. 60°, frozen and thawed 23 times	3 " "	" "	" "	" "
	4 " "	" "	" "	" "
Heated 30 min. 60°, frozen and thawed 23 times centrifuged and filtered	5 R only	R only	R only	R only
	6 " "	" "	" "	" "
Frozen and thawed 23 times, then heated 30 min. 60°	7 " "	" "	" "	" "
	8 " "	" "	" "	" "
Frozen and thawed 23 times, then heated 30 min. 60°, filtered	9 " "	" "	" "	" "
	10 " "	" "	" "	" "

In interpreting the results of this experiment attention is drawn to the following points. In the first place, transformation of type was effected only with those fractions which contained formed cell

elements. In the second place, the process of freezing and thawing was carried out under aerobic conditions. It therefore appeared possible that the factor responsible for transformation might have been destroyed by oxidation during the manipulations.

In the next experiment an attempt was made to prevent oxidation by carrying out all procedures under anaerobic conditions.

The bacteria obtained by centrifuging a culture of III S organisms were divided into two lots, A and B. Lot A was heated for 20 minutes at 60°C. A portion was then withdrawn for test and control purposes. The remainder was frozen and thawed twenty times under a heavy layer of oil. A portion of this material was withdrawn for experimental purposes and the remainder was centrifuged at high speed for 30 minutes. At the end of this time the cell-free supernatant material was pipetted off and similarly used for experimental purposes. Lot B was frozen and thawed twenty times under a heavy layer of oil and then heated for 20 minutes at 60°C. A portion was withdrawn for test purposes. The remainder was centrifuged at high speed for 30 minutes. The clear supernatant fluid was pipetted off and used in tests.

The results of this experiment may be tabulated as follows:

Suspension of S Cells

1. Heated 30 min. at 60°C.....Effective
2. Heated 30 min. at 60°C., frozen and thawed anaerobically.....Effective
3. Heated 30 min. at 60°C., frozen and thawed anaerobically, centrifuged, supernatant material.....Not effective
4. Frozen and thawed anaerobically, heated 30 min. at 60°C.....Not effective
5. Frozen and thawed, anaerobically, heated 30 min. at 60°C., centrifuged, supernatant material.....Not effective

The additional points to be noted in this experiment are: (1) the supernatant material from heat-killed, frozen and thawed bacterial suspension of S cells was not effective; (2) a suspension of S cells, frozen and thawed anaerobically and subsequently heated for 30 minutes at 60°C., was not effective. This experiment suggested that the failure to induce transformation was due to the existence of unfavorable conditions created during the disruption of living bacterial cells.

The Effect of (1) Bacterial Peroxide, (2) the pH in Cultures from Which Vaccines Are Prepared for Transformation Purposes

In the experiments carried out up to this point the observation had been made that S vaccines prepared from different lots of culture varied greatly in their capacity to induce transformation. It was noted that vaccines made from old and autolyzing cultures were quite ineffective. Moreover the procedure of subjecting the culture to a preliminary heating for 10 minutes at 60° before centrifugation

TABLE III

The Effect of (1) Bacterial Peroxide, (2) pH in Cultures from Which Vaccines Are Made for Transformation Purposes

2 R culture, 1 drop 10⁻⁶ dilution; 0.5 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine, equivalent of 50 cc. of original culture, prepared as indicated.

Age of culture	pH of culture	Bacterial peroxide	Colonies on plates streaked		
			24 hours	48 hours	72 hours
<i>hrs.</i>					
16	7.7	—	R only	R only	Numerous III S
			" "	Over half III S	Nearly all " "
24	7.3	—	" "	Numerous " "	" " " "
			" "	R only	Several " "
40	7.3	—	" "	" "	Numerous " "
			" "	Numerous III S	Nearly all " "
48	7.3	++	" "	R only	R only
			" "	" "	" "
64	7.2	+++	" "	" "	" "
			" "	" "	" "

appeared to be of value in preserving the necessary factor (4). An attempt was made to verify these observations in the following way.

A culture of III S pneumococcus in plain broth was incubated at 37°C. Samples of the culture were withdrawn at the following intervals,—16, 24, 40, 48 and 64 hours. After centrifugation the bacteria obtained from these samples were heated for 20 minutes at 60°. The supernatant material was tested for the presence of bacterial peroxide and its pH determined. The different lots of vaccine were then used in transformation experiments. The results are presented in Table III.

From the data presented in Table III it is evident that vaccines prepared from cultures of S organisms are not effective in inducing

transformation of type if bacterial peroxide is present in demonstrable amounts in the cultures. Within the limits of the experiment the pH range did not materially affect the results. The experiment was repeated and entirely similar results were obtained. This finding offered a solution to many hitherto unexplained results and suggested that the principle responsible for transformation of type was possibly susceptible to oxidizing influences. In subsequent attempts to isolate the principle responsible for transformation of type the significance of this observation was recognized. These experiments are being continued.

DISCUSSION

The study of the conditions under which transformation of pneumococcal types may be effected by *in vitro* procedures has led to a clearer understanding of the mechanism of the transformation process.

It has been shown that the nature of the R culture selected for transformation purposes materially affected the results obtained. Thus, R cultures which possessed only slight degrees of R stability were found to be the most suitable for transformation purposes. An adequate explanation of this finding cannot be offered until further knowledge is available as to the factors which determine the degree of stability possessed by various R cultures.

In considering the nature of the mechanism by which transformation of type is effected two possibilities present themselves: either a latent attribute of the R cell may be stimulated by its association with the S vaccine, or the R organisms may acquire a new property from the vaccine. The former conception involves the assumption that all R pneumococci possess at all times the latent capacity of elaborating any one of the known varieties of specific polysaccharides associated with S organisms. The latter hypothesis suggests the possibility that, at times, certain attributes of bacteria may be transferred from organisms of one type to those of another type of the same species. In the course of the present investigation no evidence has been obtained to support either hypothesis but attention is directed to certain characteristics of the property, possessed by S vaccines, which is responsible for transformation of type.

In the previous paper it was shown that the factor responsible for transformation was heat-labile, being destroyed at temperatures above 80°C. It therefore cannot be related to the specific soluble substance of pneumococcus for this has been shown to resist temperatures of 100°C. (7). Attempts to effect transformation by the use of purified specific soluble substance were uniformly unsuccessful.

The experiments described, in which attempts were made to induce transformation by cell-free extracts of S vaccines, throw further light on the nature of the property responsible for transformation of type. These experiments suggested that the essential factor was destroyed by the bacterial enzymes released during disruption of living bacterial cells. Additional evidence in support of this view was obtained in the experiments in which it was shown that vaccines prepared from old cultures in which bacterial peroxide was present in demonstrable amounts were ineffective in transformation.

In these two characteristics, thermolability and susceptibility to the action of enzymes liberated in old broth cultures, the property responsible for transformation bears a striking resemblance to the "antigenic specific substance" described by Day (8). Day showed that the antigenic specific substance of pneumococcus contains two groups, (1) a stable portion (ordinary specific substance), which reacts with immune serum, (2) an unstable, antigenic, portion, which provokes the formation of antibody. The first of these groups is resistant to heating at 100°C. and to the action of bacterial enzymes. The second group is destroyed by heating at 100°C. and by bacterial enzymes. It therefore appears possible that the factor contained in an S vaccine responsible for transformation of type is closely related to, if not identical with, the "antigenic specific substance" of pneumococcus. The nature of this antigenic group is conjectural. In many respects it resembles the enzymes of pneumococcus studied by Avery and Cullen (9). Further work is required to determine its true nature and precise properties.

The significance of the results reported in this and the preceding communication in the field of bacteriology and epidemiology is largely a matter of speculation. Further investigation is required to determine whether similar transformations of type occur under natural or disease conditions both in pneumococci and other bacterial species.

SUMMARY AND CONCLUSIONS

1. Further observations on the conditions under which transformation of pneumococcal types may be induced by *in vitro* procedures are presented.
2. R cultures possessing only slight degrees of R stability are most suitable for transformation purposes by *in vitro* procedures.
3. Vaccines prepared from cultures subjected to the action of bacterial enzymes liberated (a) in old broth cultures, (b) during mechanical disruption of young bacterial cells, are not effective in inducing transformation of type.
4. The property of an S vaccine responsible for transformation of type is not related to the specific soluble substance of pneumococcus.
5. Attempts to effect transformation of type by the use of cell-free extracts of pneumococcus have so far proved unsuccessful.

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PHENOMENON OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES: AN ANTIBODY AUXILIARY TO SERUM NEUTRALIZATION OF MENINGOCOCCUS REACTING FACTORS

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It has been shown in previous communications (1-6) that the *B. typhosus* reacting factors of the phenomenon of local skin reactivity can be specifically neutralized in multiple proportions by immune sera *in vitro* and *in vivo*. If various amounts of a toxic filtrate are titrated against a constant amount of immune serum it is possible to determine the largest amount of filtrate which is consistently neutralized (*i.e.* in all rabbits tested) by this amount of serum, and thus to express quantitatively the neutralizing potency of the serum ("CN" titer). If the titration is continued beyond the CN titer (*i.e.* in greater quantity of toxin) there is usually observed a rather protracted zone of irregular neutralization ("IN" titer) in which the percentage of positive rabbits is considerably lower than in the control group. There is evidently present in these serum-filtrate mixtures a sufficient amount of non-neutralized toxic substance to elicit reactions in rabbits of high susceptibility to the phenomenon. If still larger amounts of toxic filtrate are titrated one arrives at a point where the addition of serum produces no effect. It is clear that the CN titer,—which indicates how many units of toxic filtrate are completely neutralized by a given amount of serum,—is fairly well demarcated. The results obtained by the determination of the IN titer (*i.e.* of the largest number of units irregularly neutralized by a given amount of serum) are less accurate because of the individual fluctuations in susceptibility of normal rabbits to the phenomenon.

When considerable data had been accumulated concerning the neutralization of *B. typhosus* toxic substances and the phenomenon of

local skin reactivity had been also successfully produced with meningococcus culture filtrates (7), an attempt was made to study the neutralizing potency of antimeningococcus horse sera in order to obtain a laboratory method for the evaluation of the curative effect of these sera. It became evident from these studies, however, that among the various batches of therapeutic sera tested, none was sufficiently powerful to produce consistent neutralization similar to that obtained in the work on *B. typhosus*. It was then decided to compute the potency by the determination of the IN titer. This was done by finding the highest dilution of a serum capable of inhibiting reactions in at least three out of five rabbits injected with doses which elicited reactions in about 75 per cent of control rabbits (8). At the same time the immunization of horses was begun in an effort to develop a serum of higher neutralizing potency. The immunization consisted of subcutaneous injections of toxic filtrates and intravenous injections of heat-killed and live cultures. With considerable difficulties, sometimes shortly after the beginning of immunization, sometimes after many months, it was occasionally possible to obtain a serum which neutralized consistently a fairly large number of toxic units. In these determinations there was again adopted the above described titration of various amounts of toxic units against constant amounts of serum (*i.e.* 0.25 cc.) (9). However, further studies brought out the curious fact that in certain sera of a low CN titer the IN titer appeared quite high. Moreover, if multiples of 0.25 cc. were tested, the CN titer did not increase proportionately, a fact illustrated by the following example:

0.25 cc. of antimeningococcus horse serum H₇ Bl₂₂₀ showed complete neutralization of 15 toxic units and irregular neutralization of 40 units. 1 cc. of the same serum showed irregular neutralization of 25 units.

This is in striking contrast to observations made with other immune sera (*i.e.* antityphoid and anticoli horse sera), which showed complete neutralization in multiple proportions. It seemed possible that the inability of antimeningococcus sera to neutralize completely the toxic factors in multiple proportions may be due to the lack of some auxiliary factor, perhaps non-specific in nature. A search was made for this hypothetical auxiliary factor. This was done by studying the neutralization of meningococcus reacting factors by means of mixtures of

antimeningococcus sera with heterologous immune sera which by themselves were capable of complete neutralization in multiple proportions of their homologous toxic substances. The results of the work are embodied in this paper.

EXPERIMENTAL

Tables I and II present a summary of experiments on neutralization of the reacting factors of various serological groups of meningococcus by means of polyvalent antimeningococcus horse sera and also by means of mixtures of these sera with various normal and heterologous immune sera.

On the morning of the experiment the serum mixtures were made in proportions indicated in the tables. These were kept for from 1 to 2 hours at room temperature. The technique of neutralization experiments was the same as described before (9), except that each test was done on three instead of four rabbits. If no reactions were obtained in the three rabbits tested, the result was considered as showing consistent neutralization of reacting factors (CN). If one of three rabbits showed a reaction the result was recorded as "irregular neutralization" (IN). If two of three or all rabbits showed reactions there was recorded "no neutralization" ("NN").

As is seen from Table I, antimeningococcus horse sera tested in various amounts against filtrates of two strains of Group I, of one non-grouped patient's strain ("Fabio"), and of one Group III strain gave only irregular neutralizations. The larger the amount of serum used, the higher was the IN titer. Mixtures of the various heterologous sera indicated in the table and also of two batches of normal horse sera with the same antimeningococcus sera also showed only irregular neutralizations.

As is seen in Table II, mixtures of antimeningococcus horse sera with anticoli horse serum displayed a remarkable neutralizing potency. First, these mixtures exhibited a substantial increase in the IN titer (see Table I, Groups 2 and 11 and Table II, Groups 2 and 16); second, they showed complete neutralization of large numbers of toxic units; and, third, they differed in their potency for filtrates of various serological groups (Table II, Groups 8, 9, 10, and 27).

As illustrated by Groups 1 to 10 of Table II, it was possible to determine the CN titers of the mixtures. Thus, if a given mixture

TABLE I

Neutralization of Meningococcus Reacting Factors by Means of Mixtures of Normal and Heterologous Sera with Antimeningococcus Sera

Rabbit group No.	Antimeningococcus horse serum	Amount per kilo weight	Heterologous and normal horse sera	Amount per kilo weight	Meningococcus strain used for toxic filtrate	No. of toxic units	Results
		cc.		cc.			
1	H ₇ Bl ₁₈₉	0.33	—	—	Group I (44 D)	100	N N
2	" "	0.4	—	—	" "	100	"
3	" "	1	—	—	" "	100	I N
4	" "	1	—	—	" "	125	"
5	" "	0.35	—	—	Group I (123)	40	N N
6	" "	0.35	—	—	" "	60	"
7	" "	1	—	—	" "	50	"
8	" "	1	—	—	" "	75	"
9	" "	1	—	—	"Fabio"	100	"
10	" "	1	—	—	Group III (44 B)	90	I N
11	H ₇ Bl ₁₈₇	0.25	—	—	" "	25	"
12	" "	1	—	—	" "	40	"
13	H ₇ Bl ₂₂₀	1	—	—	Group I (44 D)	50	N N
14	H ₇ Bl ₁₈₉	0.4	Normal horse serum 176/177	0.6	" "	100	"
15	" "	0.2	" "	0.8	" "	100	"
16	" "	0.9	" "	0.1	" "	100	"
17	H ₇ Bl ₂₂₀	0.4	Normal horse serum 213	0.6	Group III (44 B)	60	"
18	" "	0.9	" "	0.8	" "	50	I N
19	H ₇ Bl ₁₈₉	0.4	Scarlet fever anti-toxin	0.6	Group I (44 D)	100	N N
20	" "	0.4	Antityphoid horse serum 16/194	0.6	" "	100	"
21	" "	0.4	Tetanus antitoxin N.Y.B.H.	0.6	" "	100	"
22	" "	0.4	Type I pneumococcus antibody N.Y.B.H.	0.6	" "	100	"
23	" "	0.5	Antityphoid horse Serum 16/194	0.5	" "	100	"
24	H ₇ Bl ₂₂₀	0.9	Human convalescent pneumonia serum	0.1	Group III (44 B)	100	"
25	" "	0.9	Antityphoid horse Serum 16/194	0.1	" "	100	"
26	" "	0.9	Type II pneumococcus antibody N.Y.B.H.	0.1	" "	100	"

TABLE II

Neutralization of Meningococcus Reacting Factors by Means of Mixtures of Anticoli Horse Serum with Antimeningococcus Horse Sera

Rab- bit group No.	Antimen- ingococcus horse serum	Amount per kilo weight	Anticoli horse serum	Amount per kilo weight	Meningococcus strain used for toxic filtrate	No. of toxic units	Results
		cc.		cc.			
1	H ₇ Bl ₁₃₉	0.4	H ₂₀ Bl ₁₃₉	0.6	Group I (44 D)	100	CN
2	" "	0.4	" "	0.6	" "	130	IN
3	" "	0.4	" "	0.6	" "	160	NN
4	H ₇ Bl ₁₃₇	0.9	" "	0.1	Group I (123)	100	CN
5	" "	0.9	" "	0.1	" "	130	NN
6	H ₇ Bl ₁₃₉	0.9	" "	0.1	"Fabio"	100	CN
7	" "	0.9	" "	0.1	" "	130	NN
8	H ₇ Bl ₂₂₀	0.4	" "	0.6	Group III (44 B)	60	CN
9	" "	0.4	" "	0.6	" "	100	"
10	" "	0.4	" "	0.6	" "	125	IN
11	—	—	" "	0.1	Group I (44 D)	8	CN
12	—	—	" "	0.1	" "	20	NN
13	—	—	" "	0.5	" "	30	CN
14	—	—	" "	0.5	" "	60	NN
15	—	—	" "	1	" "	90	CN
16	—	—	" "	1	" "	110	IN
17	—	—	" "	0.1	Group III (44 B)	15	CN
18	—	—	" "	0.1	" "	25	NN
19	—	—	" "	0.5	" "	45	CN
20	—	—	" "	0.5	" "	60	NN
21	—	—	" "	1	" "	90	CN
22	—	—	" "	1	" "	110	IN
23	H ₃ Bl ₂₂₁	0.9	" "	0.1	" "	110	CN
24	" "	0.9	" "	0.1	" "	140	NN
25	" "	0.9	" "	0.5	" "	110	CN
26	" "	0.9	" "	0.5	" "	140	NN
27	H ₇ Bl ₂₂₃	0.4	" "	0.6	Group I (44 D)	50	"
28	H ₇ Bl ₂₂₃	0.3	—	—	Group III (44 B)	6	CN
29	" "	0.3	—	—	" "	15	IN
30	" "	0.3	—	—	" "	25	NN
31	" "	0.6	—	—	" "	10	CN
32	" "	0.6	—	—	" "	25	NN
33	" "	0.9	—	—	" "	20	IN
34	" "	0.9	—	—	" "	36	"
35	" "	1	—	—	" "	50	NN
36	" "	0.3	H ₂₃ Bl ₁₁₃	0.1	" "	8	IN
37	" "	0.3	" "	0.1	" "	15	NN
38	" "	0.6	" "	0.1	" "	12	CN
39	" "	0.6	" "	0.1	" "	20	NN
40	" "	0.9	" "	0.1	" "	25	CN
41	" "	0.9	" "	0.1	" "	30	"
42	" "	0.9	" "	0.1	" "	40	IN
43	" "	0.9	" "	0.1	" "	18	"
44	" "	0.9	" "	Dilution 1:3 0.1 cc. Dilution 1:5	" "	25	NN

neutralizes completely 100 toxic units and only irregularly 130 units, the CN titer is approximately 115 units. The CN determinations, as mentioned above, are considerably more accurate than the IN titrations.

Table II also includes data on the reciprocal relationships between the anticoli and antimeningococcus horse sera.

As is seen from Groups 11 to 22, the anticoli horse serum was by itself capable of giving complete neutralization of meningococcus reacting factors. The neutralizing property of this serum, however, differed in several respects from the specific antibody of antimeningococcus serum:—It had no agglutinins and precipitins for meningococcus (unrecorded experiments); it neutralized the toxic factors of both serological Groups I and III to the same degree; and it was capable of complete neutralization in multiple proportions.

The next step was to determine whether the high complete neutralization obtained with the mixtures described was due to summation of neutralizing titers of sera making up the mixtures.

Inasmuch as 0.1 and 0.5 cc. of anticoli horse Serum H₆₀ Bl₁₈₈ contain about 15 and 45 CN units, respectively, it follows that, should there be summation of titers, then a mixture consisting of 0.9 cc. of antimeningococcus Serum H₈ Bl₂₅₁ and 0.5 cc. of Serum H₆₀ Bl₁₈₈ must have a higher titer than a mixture consisting of the same amount of the antimeningococcus serum and only 0.1 cc. of Serum H₆₀ Bl₁₈₈. However, as is seen from Groups 23 to 26, both mixtures remained of the same titer. Similarly, in case of summation of titers, any mixture containing 0.6 cc. of anticoli horse Serum H₆₀ Bl₁₈₈ should have at least 50 neutralizing units irrespective of the titer of the antimeningococcus serum. But, as is seen from Groups 1 to 3 and 27, a mixture consisting of 0.4 cc. of antimeningococcus Serum H₇ Bl₁₈₉ and 0.6 cc. of anticoli horse Serum H₆₀ Bl₁₈₈ gave a CN titer of 115 units, while a mixture consisting of 0.4 cc. of antimeningococcus Serum H₇ Bl₂₂₀ and 0.6 cc. of the same anticoli serum gave no neutralization of 50 units. As is seen from Groups 3 and 4 of Table I and Group 35 of Table II, the antimeningococcus Serum H₇ Bl₁₈₉ had a higher IN titer than Serum H₇ Bl₂₂₀.

From the data thus far presented, therefore, the following conclusions can be drawn:

1. The high CN titer of mixtures of antimeningococcus and anticoli sera is not due to mere summation of titers of both sera.
2. The addition of anticoli serum to antimeningococcus serum of a low neutralizing potency does not bring about any increase in potency

of the mixture. Moreover, the independent neutralizing potency of the anticoli serum seems to become abolished altogether when mixed with an antimeningococcus serum poor in neutralizing antibodies.

3. The addition of anticoli serum to an antimeningococcus serum of a high IN titer results in a high CN titer of the mixture.

These conclusions can be further supported by experiments with Groups 28 to 44 of Table II.

In these experiments there was tested the neutralizing potency of various amounts of antimeningococcus Serum H₇ Bl₂₀₂ and of mixtures of this serum with diluted and undiluted anticoli horse Serum H₆₀ Bl₁₈₈. As could be expected, various amounts of antimeningococcus serum alone gave irregular neutralizations except when tested with small numbers of toxic units. The addition of 0.1 cc. of anticoli serum previously diluted 1:3 had no effect on neutralizing potency. Mixtures of amounts as small as 0.3 and 0.6 cc. of antimeningococcus serum with 0.1 cc. of undiluted anticoli serum did not result in any noticeable increase of the CN titer. In spite of the fact that the latter amount of anticoli serum could neutralize completely about 15 toxic units and 0.3 and 0.6 cc. of antimeningococcus serum could neutralize completely 10 and 15 units, respectively, the mixture of 0.3 cc. of antimeningococcus serum with 0.1 cc. of undiluted anticoli serum neutralized completely less than 8 units, and the mixture of 0.6 cc. of antimeningococcus serum with the above amount of anticoli serum neutralized completely only 15 units, *i.e.* the same number of units which could be neutralized independently by either ingredient of the mixture. However, when 0.1 cc. of undiluted anticoli serum was added to 0.9 cc. of antimeningococcus serum, the mixture gave complete neutralization of 35 toxic units, although this amount of antimeningococcus serum gave independently only irregular neutralization of 20 units.

From these observations it is seen again that the anticoli serum does not exhibit its independent neutralizing potency when mixed with antimeningococcus serum containing neutralizing antibodies of low concentration (0.3 cc. of H₇ Bl₂₀₂); that it produces no effect when added to a somewhat larger amount of antimeningococcus antibodies (*i.e.* 0.6 cc. of H₇ Bl₂₀₂); and that it converts (beyond its own neutralizing potency) irregular neutralization into complete neutralization when added to a sufficient concentration of antimeningococcus antibodies capable of irregular neutralization. For these reasons it may be assumed that the anticoli serum contributes some auxiliary factor to the specific antibody-antigen combination and enters with it into an intricate relationship.

Further studies were made in order to define closer the nature of the auxiliary factor in the anticolli horse serum.

The following horse sera were tested for complement and anticomplementary properties, namely: anticolli Serum $H_{60}Bl_{188}$; normal Serum 176/177; antimeningococcus Sera H_7Bl_{187} , H_7Bl_{189} , and H_7Bl_{202} .

Amounts of each serum tested for complement were 0.01, 0.05, 0.1, and 0.2 cc. These amounts were each mixed with 2 units of sheep red blood cell "amboceptor" (*i.e.* 0.1 cc. of 1:50 dilution) and 0.5 cc. of 5 per cent suspension of sheep red blood cells. The mixtures were incubated in a water bath at 37° for 30 minutes. No hemolysis occurred in any of the tubes.

The same sera in above mentioned amounts were tested for anticomplementary property in two ways:

Test I.—The various amounts of sera were each mixed with 1 unit of guinea pig complement. After incubation in water bath at 37° for 30 minutes there was added to each of the mixtures 0.5 cc. of a 5 per cent suspension of sheep red blood cells and 2 units of sheep red blood cell "amboceptor." The new mixtures were then incubated in water bath at 37° for 20 minutes. All the sera proved to be anticomplementary in 0.1 and 0.2 cc. amounts. Sera 176/177 and $H_{60}Bl_{189}$ were slightly less anticomplementary than the others.

Test II.—In this test the various amounts of sera were mixed each with 1 unit of guinea pig complement and 0.2 cc. of beef heart antigen used for Wassermann tests. The rest of the technique and the results were the same as in Test I.

Heating of the sera at 56° for 30 minutes completely removed the anticomplementary property.

These experiments established the fact that the auxiliary factor is not in the nature of complement and has no apparent relation to the anticomplementary property of sera.

In Table III there are summarized tests for the auxiliary factor in various bleedings of several horses immunized with *B. coli* cultures and culture filtrates. These tests were carried out in the manner described on page 713.

The sera tested were added to a given antimeningococcus serum in the proportion 1:10. Two antimeningococcus sera, H_7Bl_{202} and H_7Bl_{220} , were employed alternately. The amounts of Group III toxic filtrate used were 35 and 90 units respectively, completely neutralized by mixtures of these sera with anticolli horse serum $H_{60}Bl_{153}$.¹ (See Table II.)

¹The CN titer of 9 parts of H_7Bl_{202} + 1 part $H_{60}Bl_{153}$ was 35 units. The CN titer of mixture of 9 parts of H_7Bl_{220} + 1 part $H_{60}Bl_{153}$ exceeded 90 units.

The horses were immunized as follows:

Horse 20.—Immunization began 7/26/29. The first dose was a subcutaneous injection of 10 cc. of "*B. coli* polyvalent toxin." The latter consisted of a mixture of filtrates of 5 day old tryptic digest broth cultures of fourteen strains. Two strains were rough. The remaining strains were smooth. 0.2 cc. of potassium alum was added to the filtrate before injection. All the injections were made at weekly intervals. The first 2 months the horse received only subcutaneous injections. The doses were gradually increased so that on 9/26/29 there was injected subcutaneously 200 cc. of the filtrate. From this date the horse was immunized weekly both subcutaneously with the filtrate and also intravenously with "polyvalent heat-killed *B. coli* vaccine." The latter consisted of a mixture of the same strains employed for preparation of the filtrates. The first intravenous dose was 100,000,000 organisms. There was also a gradual increase with each subsequent injection. On 11/26/29 the intravenous dose of one-half billion organisms was reached. The subcutaneous doses fluctuated from 150 to 200 cc. during the period between 9/26 and 11/26/31. Bleeding 95 was made 11/25/31. The immunization was continued in a similar manner until May, 1930, the subcutaneous doses fluctuating between 150 and 200 cc. and the intravenous doses between one-half billion and one billion organisms. Horses 60, 2, 8, and 148 were immunized for various periods of time in a manner similar to Horse 20. During the course of immunization Horse 60 rested a few times, for several weeks each time—as indicated in Table III.

Horse 101.—Immunization of this horse was less intensive. It began 9/29/31 and continued at weekly intervals. The first subcutaneous dose was 5 cc. of the "polyvalent *B. coli* toxin." The subcutaneous doses were gradually increased so that 4/2/31 the horse received 20 cc. of the filtrate. From 4/9 to 5/9/31 the horse received both subcutaneous injections of the "polyvalent *B. coli* toxin" and intravenous injections of the "polyvalent heat-killed *B. coli* vaccine." The subcutaneous dose of 85 cc. was reached on 5/9/31. The intravenous doses were also gradually increased with each injection. The first intravenous dose was 300,000,000 organisms. On 5/9/31 the horse received intravenously 800,000,000 organisms.

As is seen from Table III, the auxiliary factor described is in the nature of an antibody, the production of which is stimulated by immunization with *B. coli* heated cultures and culture filtrates.

The antibody appears shortly after the beginning of immunization. The length of time necessary, however, for its development differs in horses under the same treatment. Thus, after 10 weeks of immunization the antibody was absent in Horse 2, but already present in Horse 8. The majority of horses developed the antibody in 8 to 10 weeks. The antibody also disappeared in Horses 20 and 60 during the process

TABLE III

Auxiliary Factor in Horses Immunized with B. coli Cultures and Culture Filtrates

No. of meningococcus Group III toxic units	Antimeningococcus horse serum	Bleedings of anticol horses	No. of weeks of immunization and rest	No. of days after last injection bleeding obtained	Result
90	H ₇ Bl ₂₂₀	H ₂₀ Bl ₉₅	17, immuniz.		
90	" "	H ₂₀ Bl ₁₁₆	31, "	6	-*
90	" "	H ₂₀ Bl ₁₃₈	44, "	6	+**
90	" "	H ₆₀ Bl ₁₆₉	12, "	6	-
90	" "	H ₆₀ Bl ₁₈₈	3, rest	21	+
35	H ₇ Bl ₂₀₂	H ₆₀ Bl ₂₀₁	19, immuniz.,		
90	H ₇ Bl ₂₂₀	H ₇ Bl ₂₀₉	3, rest	6	+
90	" "	H ₆₀ Bl ₂₁₇	24, immuniz.,	6	+
90	" "	H ₆₀ Bl ₂₃₀	3, rest	6	+
35	H ₇ Bl ₂₀₂	H ₆₀ Bl ₂₆₀	28, immuniz.,	6	+
35	" "	H ₆₀ Bl ₂₆₆	3, rest	6	+
35	" "	H ₆₀ Bl ₂₉₀	31, immuniz.,	6	-
90	H ₇ Bl ₂₂₀	H ₁₄₈ Bl ₉₇	3, rest	21	-
90	" "	H ₃₀ Bl ₈₅	33, immuniz.,	28	-
90	" "	H ₃₀ Bl ₁₁₇	8, rest	6	+
90	" "	H ₂ Bl ₂₀₅	33, immuniz.,	6	+
35	H ₇ Bl ₂₀₂	H ₈ Bl ₂₀₆	9, rest	6	+
35	" "	H ₁₀₁ Bl ₂₁₂	36, immuniz.,	6	+
35	" "	H ₁₀₁ Bl ₂₅₇	9, rest	6	+
35	" "	H ₁₀₁ Bl ₂₆₂	12, immuniz.	6	+
35	" "	H ₁₀₁ Bl ₂₆₇	6, "	6	+
35	" "	H ₁₀₁ Bl ₂₇₇	26, "	6	+
35	" "	H ₁₀₁ Bl ₂₉₃	10, "	6	+
35	" "	H ₁₀₁ Bl ₂₉₃	10, "	6	+
35	" "	H ₁₀₁ Bl ₂₉₃	Bleeding before immuniz.	6	+
35	" "	H ₁₀₁ Bl ₂₉₃	3, immuniz.	0	-
35	" "	H ₁₀₁ Bl ₂₉₃	4, "	6	-
35	" "	H ₁₀₁ Bl ₂₉₃	5, "	6	-
35	" "	H ₁₀₁ Bl ₂₉₃	7, "	6	-
35	" "	H ₁₀₁ Bl ₂₉₃	8, "	6	-
35	" "	H ₁₀₁ Bl ₂₉₃	9, "	6	+
35	" "	H ₁₀₁ Bl ₂₉₃		6	+

* - No auxiliary factor, since only irregular or no neutralization obtained.

** + Auxiliary factor present since complete neutralization obtained.

of immunization. After the disappearance of the antibody, Horse 60 was rested intermittently for 9 weeks. 3 weeks of immunization following the last period of 3 weeks rest again stimulated the production of the antibody.

The question of what happens to the anticoli antibody simultaneously with these fluctuations in auxiliary antibody will be taken up subsequently.

DISCUSSION AND CONCLUSIONS

The present paper embodies data concerning a certain antibody auxiliary to neutralization of meningococcus reacting factors.

The auxiliary antibody is not complement and has no apparent relationship to the anticomplementary property of horse sera.

The antibody is produced by immunization of horses with *B. coli* heat-killed cultures and culture filtrates. It appears shortly after the beginning of immunization and it may disappear during the process of immunization. Horses in which the antibody disappears can regain their power of producing it after a few weeks of immunization, following a period of rest.

The auxiliary antibody is absent from other heterologous and normal horse sera thus far tested.

When the auxiliary antibody is mixed *in vitro* with an antimeningococcus horse serum which by itself is capable of irregular neutralization in a high titer the effect is as follows:

The IN titer of the mixture is increased; the mixture acquires the power of complete neutralization (CN titer) of large numbers of toxic units; it differs in its potency for filtrates of various serological groups.

However, the auxiliary antibody does not exhibit its effect in mixtures with antimeningococcus sera unless the latter contain neutralizing antibodies of sufficient potency and concentration.

The employment of the auxiliary antibody enables one to express the neutralizing value of antimeningococcus sera in terms of complete neutralization and thus gives an accurate method for their comparative evaluation.

It has been shown in previous communications that the potency of toxic filtrates fluctuates in storage. This considerably handicaps the

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90	H ₇ Bl ₂₂₀	H ₂₀ Bl ₉₅	17, immuniz.		
90	" "	H ₂₀ Bl ₁₁₆	31, "		
90	" "	H ₂₀ Bl ₁₃₈	44, "	6	—*
90	" "	H ₆₀ Bl ₁₆₉	12, "	6	+**
90	" "	H ₆₀ Bl ₁₈₈	3, rest	6	—
35	H ₇ Bl ₂₀₂	H ₆₀ Bl ₂₀₁	19, immuniz.,	21	+
90	H ₇ Bl ₂₂₀	H ₇ Bl ₂₀₉	3, rest	6	+
90	" "	H ₆₀ Bl ₂₁₇	24, immuniz.,	6	+
90	" "	H ₆₀ Bl ₂₃₀	3, rest	6	+
35	H ₇ Bl ₂₀₂	H ₆₀ Bl ₂₆₀	26, immuniz.,	6	+
35	" "	H ₆₀ Bl ₂₆₆	3, rest	6	—
35	" "	H ₆₀ Bl ₂₉₀	33, immuniz.,	21	—
90	H ₇ Bl ₂₂₀	H ₁₄₈ Bl ₉₇	8, rest	28	—
90	" "	H ₃₀ Bl ₈₅	33, immuniz.,	6	+
90	" "	H ₃₀ Bl ₁₁₇	9, rest	6	+
90	" "	H ₂ Bl ₂₀₅	36, immuniz.,	6	—
35	H ₇ Bl ₂₀₂	H ₃ Bl ₂₀₈	9, rest	6	+
35	" "	H ₁₀₁ Bl ₂₄₂	12, immuniz.	6	—
35	" "	H ₁₀₁ Bl ₂₅₇	6, "	6	+
35	" "	H ₁₀₁ Bl ₂₆₂	26, "	6	—
35	" "	H ₁₀₁ Bl ₂₆₇	10, "	6	+
35	" "	H ₁₀₁ Bl ₂₇₇	10, "	6	—
35	" "	H ₁₀₁ Bl ₂₈₅	Bleeding before immuniz.	6	—
35	" "	H ₁₀₁ Bl ₂₉₃	3, immuniz.	0	+
			4, "	6	—
			5, "	6	—
			7, "	6	—
			8, "	6	—
			9, "	6	+
				6	+

* — No auxiliary factor, since only irregular or no neutralization obtained.

** + Auxiliary factor present since complete neutralization obtained.

of immunization. After the disappearance of the antibody, Horse 60 was rested intermittently for 9 weeks. 3 weeks of immunization following the last period of 3 weeks rest again stimulated the production of the antibody.

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The present paper embodies data concerning a certain antibody auxiliary to neutralization of meningococcus reacting factors.

The auxiliary antibody is not complement and has no apparent relationship to the anticomplementary property of horse sera.

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The auxiliary antibody is absent from other heterologous and normal horse sera thus far tested.

When the auxiliary antibody is mixed *in vitro* with an antimeningococcus horse serum which by itself is capable of irregular neutralization in a high titer the effect is as follows:

The IN titer of the mixture is increased; the mixture acquires the power of complete neutralization (CN titer) of large numbers of toxic units; it differs in its potency for filtrates of various serological groups.

However, the auxiliary antibody does not exhibit its effect in mixtures with antimeningococcus sera unless the latter contain neutralizing antibodies of sufficient potency and concentration.

The employment of the auxiliary antibody enables one to express the neutralizing value of antimeningococcus sera in terms of complete neutralization and thus gives an accurate method for their comparative evaluation.

It has been shown in previous communications that the potency of toxic filtrates fluctuates in storage. This considerably handicaps the

studies on neutralizing antibodies of sera. Inasmuch as in recent observations the CN titer of mixtures of antimeningococcus sera with the auxiliary antibody appeared to be quite stable,² an opportunity presents itself to titrate the potency of filtrates against a "stock" standard serum and thus determine the toxic titer of filtrates more accurately than by direct titrations.

It remains to be seen whether the reinforcement of antimeningococcus horse sera by means of the auxiliary antibody increases their therapeutic value.

This paper also includes data on a non-specific neutralizing factor of the following characteristics:

1. It is present in a heterologous immune serum (*i.e.* anticoli horse serum).
2. It is not associated with specific antimeningococcus antibodies (*i. e.* agglutinins and precipitins).
3. It apparently displays no serological group specificity.
4. It gives complete neutralization of meningococcus reacting factors in multiple proportions.
5. Its neutralizing effect is not displayed when mixed with an antimeningococcus serum of low potency.
6. It is not complement and not associated with anticomplementary properties of horse sera.

These properties have to be differentiated from those of the specific neutralizing antibodies of antimeningococcus sera (8, 9). The latter are stimulated by immunization with meningococcus antigens; they exhibit species, serological group, and variant specificity; they are obtained in high potency with considerable difficulties; and in most instances they neutralize completely only small numbers of toxic units, but are capable of incomplete neutralization of considerable amounts of toxic filtrates. When multiples of a given amount of antimeningococcus serum are tested there is a proportionate increase in the titer of irregular neutralization but no proportionate increase in the CN titer.

Work is in progress to determine the nature of the non-specific neutralizing factor and also to establish its relations to the auxiliary antibody.

²The mixtures were always made the day of the experiment.

SUMMARY

In this paper there is described an antibody auxiliary to the neutralization of meningococcus reacting factors. The presence of the antibody facilitates studies on neutralizing potency of antimeningococcus sera.

There is also reported a non-specific neutralizing factor of a heterologous immune serum, which can be differentiated from specific neutralizing antibodies of antimeningococcus sera. Its nature and connection with the auxiliary antibody remain to be determined.

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A SEROLOGICAL STUDY OF THE POLYSACCHARIDES OF
MENINGOCOCCUS, B. ANTHRACIS, B. PROTEUS,
B. SUBTILIS AND B. MESENTERICUS

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Since the initial studies of Heidelberger, Goebel and Avery (1) on cross-reactions between the specific polysaccharides of Pneumococcus Type II and Friedländer bacillus, the literature has contained little or nothing on reactions of similar nature between the specific polysaccharides of other organisms. However, in the study of bacterial polysaccharides, we have found many interesting cross-reactions that by theory would not have been expected. Among these, the one here reported is of special interest, from both a theoretical as well as a practical point of view. Przemycki (2) obtained a specific carbohydrate substance from the *B. proteus* organism; Furth and Landsteiner (3) also obtained a carbohydrate from *B. proteus* which they found to be specific. Combiesco, Soru and Stamatesco (4) have described a specific carbohydrate obtained from anthrax, and some of the chemical properties, such as rotation, presence of pentoses, hexoses, etc.

The wide difference between the meningococci and the Gram-positive bacilli would not lead one to suspect an immunological relationship between the carbohydrates of the two groups. Our first observation was accidental, and occurred when tests were made to determine the specificity of the anthrax polysaccharide with various immune sera. In the case of the antimeningococcus serum, we observed that an immediate precipitate formed, which increased after incubation. This was repeated with many different samples of antimeningococcic sera. We then proceeded to study the reciprocal test, i.e., meningococcus polysaccharide with anthrax serum and

found that precipitation took place. The titration of the polysaccharide from these organisms, as well as others (*B. mesentericus*, *E. proteus* and *B. subtilis*) was made, using antimeningococcus, antianthrax and antiproteus sera. The results of this test are shown

TABLE I

Titration of Polysaccharide of B. anthrax, B. proteus, B. subtilis and Meningococcus with Antianthrax, Antimeningococcus and Antiproteus Sera

Final dilution of polysaccharide	Antimeningococcus serum (6383) undiluted				Antianthrax serum (8795) undiluted				Antiproteus serum (6202) undiluted			
	1/40,000	1/160,000	1/640,000	1/1,200,000	1/20,000	1/80,000	1/320,000	1/1,200,000	1/2000	1/8000	1/16,000	1/64,000
Meningococcus.....	4	4	4	4	3	1	1	0	2	2	0	0
Anthrax.....	4	4	4	3	4	4	4	1	0	0	0	0
Proteus.....	4	4	4	3	3	1	0	0	4	4	4	3
Mesentericus.....	4	4	4	4	4	2	0	0	0	0	0	0
Subtilis.....	4	4	4	3	3	1	0	0	2	1	0	0

Overnight readings.

TABLE II

Titration of Antimeningococcic, Antianthrax and Antiproteus Sera with Different Polysaccharides (1/10,000)

Final dilution of serum	Antimeningococcic serum (8363)						Antianthrax serum (8795)						Antiproteus serum (6206) rabbit					
	1/2	1/4	1/8	1/16	1/32	1/64	1/2	1/4	1/8	1/16	1/32	1/64	1/2	1/4	1/8	1/16	1/32	1/64
Meningococcus..	4	4	4	2	0	0	3	2	1	0	0	0	2	1	0	0	0	0
Anthrax.....	4	4	4	2	0	0	4	4	4	4	2	0	0	0	0	0	0	0
Proteus.....	4	4	4	2	0	0	3	2	1	0	0	0	4	4	4	4	3	2
Mesentericus....	4	4	4	1	0	0	4	2	1	0	0	0	0	0	0	0	0	0
Subtilis.....	4	4	4	2	0	0	3	2	1	0	0	0	2	1	0	0	0	0

in Table I. We found that, with the antimeningococcic serum, all of the polysaccharides gave more or less the same result, while with antianthrax serum the differences were more marked, showing the anthrax polysaccharide to be more potent and specific in higher dilutions. We then titrated the same sera and a *proteus* antiserum

against the different polysaccharides in order to see if the amount of antibody precipitated by the different carbohydrates varied. The results of this test are given in Table II, according to which the meningococcus, *proteus* and *subtilis* polysaccharides are shown to behave in the same manner as far as the antimeningococcus serum is concerned, while the homologous polysaccharide with the antianthrax serum acts specifically in the higher dilutions of serum, the other four polysaccharides reacting similarly toward each other. The homologous polysaccharide with antiproteus serum is specific in higher dilutions of the former, the meningococcus and *subtilis* acting only at lower dilutions and the anthrax and *mesentericus* negatively. The antimeningococcus serum has the broadest antigenicity, being pre-

TABLE III
Agglutination Tests with Anthrax, Meningococcus and Proteus Antigens with Antimeningococcus, Antianthrax and Antiproteus Sera

Final dilution of serum	Antimeningococcus serum (8363)						Antianthrax serum (8795)						Antiproteus serum (6206)					
	1/20	1/10	1/80	1/160	1/320	1/640	1/160	1/320	1/640	1/1280	1/2500	1/5000	1/10	1/10	1/80	1/160	1/640	1/2500
Meningococcus..	4	4	4	4	4	3	3	3	2	2	0	0	4	4	3	1	0	0
Anthrax.....	4	4	4	3	2	2	4	4	4	4	3	2	0	0	0	0	0	0
Proteus.....	4	4	3	1	0	0	0	0	0	0	0	0	4	4	4	4	4	2

cipitated by all the heterologous polysaccharides used. With the anthrax serum, we found specificity of the homologous carbohydrate in the higher dilutions of the carbohydrate, while the other four acted similarly. Finally with the antiproteus serum we found specificity of its homologous polysaccharide in high dilutions and some similarity to the *subtilis* and meningococcus.

Agglutination tests were made with meningococcus, anthrax and *proteus* against antimeningococcus, antianthrax and antiproteus sera. The results are shown in Table III. In this test we confirmed the observations obtained in a previous test with the polysaccharide, the meningococcus remaining a broad antigen for the three sera, while anthrax and *proteus* were more limited. It is of interest to note the polyvalency of the sera as well as of the antigens, for this may have

some bearing on the agglutination in typhus cases with *proteus* antigen, since in some typhus sera we have obtained agglutination with the meningococcus antigen. We will report these studies in a future paper.

In order to secure more information, we made absorption tests with antimeningococcus and antianthrax sera and the various polysaccharides, performing an agglutination test with the homologous organism of the antiserum as well as a precipitation test with the homologous polysaccharide, to determine whether antibody was absorbed by the specific carbohydrate. For this experiment, we had to use concentrated sera prepared according to Felton's (5) water precipitate method, in order to test the dilution obtained in the absorption process and still possess carbohydrate precipitable substance. The original serum showed precipitin in dilution 1/8, while in the concentrated preparation it gave a titer of 1/64.

Method

To 2 cc. of concentrated serum, were added 2 cc. of the polysaccharide 1/1000, incubated for 2 hours at 37°C., and placed in the ice box overnight. The mixture was centrifuged and from the supernatant liquid, 2 cc. were taken and absorbed again with the polysaccharide. This procedure was repeated four times. In each sample the precipitin and agglutination tests were done with the homologous polysaccharide and antigen.

The results of absorption of antimeningococcus serum are shown in Table IV. Here we noticed that the carbohydrate antibody was equally well absorbed by all of the polysaccharides used, while the agglutination titer of the serum was unaffected. In Table V we show the results of absorption of antianthrax serum. We note here that while the heterologous polysaccharides gave a precipitate with this serum, only the homologous polysaccharide absorbed the anthrax specific antibody. Here we have at least two anticarbohydrate antibodies, one specific, easily absorbed by its specific carbohydrate and another probably group specific as those found in antipneumococcus serum or even more broadly non-specific. Yet the agglutination titer of the serum is not changed by any of the absorbing polysaccharides, not even when the specific polysaccharide absorbs its homologous antibody.

The absorption tests with antiproteus serum only will be mentioned because the amount of immune serum was limited and no concentration was obtained, although the meningococcus and *subtilis* poly-

TABLE IV

Precipitin Tests with Meningococcus Polysaccharide 1/10,000, after Absorption of Antimeningococcic Serum with Meningococcus, Anthrax, Proteus, Subtilis and Mesentericus Polysaccharide 1/1000, as Well as the Agglutination Titer of the Same Serum

Final dilution			1/4	1/8	1/16	1/32	1/64	1/128	Agglutination titer
Absorbed with meningococcus polysaccharide 1/1000	1st		1	0	0	0	0	0	3:1/500
	2nd		—	0	0	0	0	0	
	3rd		—	—	0	0	0	0	
	4th		—	—	—	0	0	0	
Absorbed with anthrax polysaccharide 1/1000	1st		1	0	0	0	0	0	3:1/500
	2nd		—	0	0	0	0	0	
	3rd		—	—	0	0	0	0	
	4th		—	—	—	0	0	0	
Absorbed with <i>subtilis</i> polysaccharide 1/1000	1st		2	1	0	0	0	0	3:1/500
	2nd		—	1	0	0	0	0	
	3rd		—	—	0	0	0	0	
	4th		—	—	—	0	0	0	
Absorbed with <i>mesentericus</i> polysaccharide 1/1000	1st		2	1	0	0	0	0	3:1/500
	2nd		—	1	0	0	0	0	
	3rd		—	—	0	0	0	0	
	4th		—	—	—	0	0	0	
Absorbed with <i>proteus</i> polysaccharide 1/1000	1st		1	0	0	0	0	0	3:1/500
	2nd		—	0	0	0	0	0	
	3rd		—	—	0	0	0	0	
	4th		—	—	—	0	0	0	
Control serum with salt solution	1st		4	4	4	4	4	2	3:1/500
	4th		—	—	—	4	4	2	

saccharides did not absorb the specific precipitable substance for the *proteus* polysaccharide showing the same non-specific phenomenon as was found in the absorption experiments with antianthrax serum. The *proteus* polysaccharide is specific.

DISCUSSION

The interesting findings of Heidelberger, Goebel and Avery on the immunological and chemical similarity between the polysaccharides obtained from a strain of *Bacillus friedlaenderi* and from Pneumo-

TABLE V

Precipitin Tests with Anthrax Polysaccharide (1/10,000) after Absorption of Anti-anthrax Serum with Meningococcus, Anthrax, Proteus, Mesentericus and Subtilis, as Well as the Agglutination Titer of the Same Serum

Final dilution of serum			1/4	1/8	1/16	1/32	1/64	1/128	Agglutination titer
Absorbed with meningo-coccus polysaccharide 1/1000	1st		4	4	4	4	3	2	3:1/5000
	2nd		—	4	4	4	3	3	
	3rd		—	—	4	4	2	2	
	4th		—	—	—	4	2	1	
Absorbed with anthrax polysaccharide 1/1000	1st		2	2	1	1	0	0	3:1/5000
	2nd		—	2	0	0	0	0	
	3rd		—	—	0	0	0	0	
	4th		—	—	—	0	0	0	
Absorbed with <i>subtilis</i> polysaccharide 1/1000	1st		4	4	4	4	4	3	3:1/5000
	2nd		—	4	4	4	4	3	
	3rd		—	—	4	4	4	2	
	4th		—	—	—	4	4	2	
Absorbed with <i>mesentericus</i> polysaccharide 1/1000	1st		4	4	4	4	4	3	3:1/5000
	2nd		—	4	4	4	4	3	
	3rd		—	—	4	4	4	2	
	4th		—	—	—	4	4	2	
Absorbed with <i>proteus</i> polysaccharide 1/1000	1st		4	4	4	4	4	2	3:1/5000
	2nd		—	4	4	4	4	3	
	3rd		—	—	4	4	4	3	
	4th		—	—	—	4	4	2	
Control serum with salt solution	1st		4	4	4	4	4	4	3:1/5000
	4th		—	—	—	4	4	2	

coccus Type II, are somewhat similar to our findings with these other organisms. The possibility of a similar chemical composition will be left for future study. Again the possibility arises of having in the

polysaccharides of these organisms a similar or common substance to all of them, as was found in the pneumococcus by Tillett, Goebel and Avery (6).

From previous work and from our present findings, we are aware of the complexity of bacterial antigens and of the necessity for a chemical structural study of the different polysaccharides. It is of interest to remember that while *B. anthracis* and *B. mesentericus* are so different in their biological behavior, Cowles (7) has found a bacteriophage common to both, suggesting a related chemical structure; a finding corroborated by our polysaccharide studies.

The work of Heidelberger, Goebel and Avery with *B. friedlaenderi* and Pneumococcus Type II has been used in detecting an immunological method making it possible to predict chemical similarities in the carbohydrate as Landsteiner (8) has suggested for the erythrocytes in the different species of animals.

CONCLUSIONS

1. The meningococcus polysaccharide reacts with a broad precipitable carbohydrate antibody in common with those of *B. anthracis*, *B. subtilis*, *B. proteus* and *B. mesentericus*.

2. The anthrax and *proteus* polysaccharides are specific in the higher dilutions of serum.

3. Antianthrax serum contains two different polysaccharide precipitable antibodies, one specific and the other non-specific.

4. Agglutinins have no relation to the carbohydrate precipitable substance, specific or non-specific.

5. An immunological method is given for the study of the probable chemical relation or similarity of polysaccharides of different bacteria similar to that given by Heidelberger, Goebel and Avery from a strain of Friedländer bacillus and Pneumococcus Type II.

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ELECTROPHORESIS EXPERIMENTS WITH THE VIRUS AND PROTECTIVE BODIES OF YELLOW FEVER*

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Much valuable information has come to light in recent years through the application of the knowledge and technique of colloidal chemistry to the problems involved in the study of the ultramicroscopic viruses. Among the important discoveries made has been the fact that many viruses migrate in an electric field in much the same manner as toxins, antitoxins, bacteria, and other minute particles in suspension. Differences in the electrical behavior of a number of viruses under similar conditions would seem to indicate that the migration is due in each case to some specific property of the infectious agent. It is recognized, however, that electrophoresis is influenced by a number of factors external to the migrating particles. For example, there are good reasons for supposing the viruses to be closely, even inseparably, associated with proteins of their hosts and that electrophoresis may be modified by this association. In the present state of our knowledge, however, this does not detract from the value of electrical experiments. Constant association with some particular serum constituent may be due to some special peculiarity of a given virus, the serum constituent conferring upon the virus an apparently constant property demonstrable by exposure to an electric current.

The electrical properties of the virus of vaccinia and of various races of bacteriophage have received much attention, especially from Douglas and Smith (1), Todd (2), Yaoi and Kasai (3), Krueger, Ritter, and Smith (4), and other workers.

* The phenomenon of the migration of colloidal particles under the influence of the electric current is known as electrophoresis; the terms anaphoresis and cathaphoresis are used for migration to the anode and cathode respectively (15).

The viruses of fowl-pox, myxoma of rabbits, and encephalitis of rabbits, as well as bacteriophage, have recently been very carefully studied by Natarajan and Hyde (5), with interesting results. It has been suggested that separation of certain viruses from extraneous material may eventually be accomplished by means of electrophoresis; but all workers are not of the same opinion upon this point. A possible step in this direction, however, has been made by Olitsky and Long (6) in the recovery of vaccinia virus from non-infective tissues of recovered rabbits and from neutral serum-virus mixtures. In discussing the work of Brown and Broom, Hindle and Findlay (7) point out that a knowledge of the charge of the yellow fever virus may serve as a rational basis for therapy of the disease. It is to be hoped that additional knowledge of the electrical and other physical properties of the ultramicroscopic viruses may lead to further advance.

While the electrical charge of a virus may be inferred, in some instances, from adsorption or filtration experiments, it must be borne in mind that adsorption and filtration, although related, are probably not entirely dependent upon electrical forces. As pointed out in the preceding paragraph, even results obtained electrically with an apparatus of the Landsteiner and Pauli (8) type, or one of its numerous modifications, may not depend solely upon the electrical charge (if any) of the virus itself. In considering the electrical charge of viruses one must guard constantly against errors due to associations between the virus and other substances; and methods involving agar bridges must include precautions against undue electroendosmotic flow. The volume of such flow through 2 per cent agar bridges 15 to 20 cm. in length is very small (9) with a current of 5 ma. (110 volts). In the "U" tube type of apparatus of 50 to 60 cc. capacity it probably does not affect the results, especially since the agar is discarded. However, where the agar itself is used as a test source of the material under investigation and has been in contact with it during the course of the experiment, electro-osmosis must be considered in interpreting the results. Very rapid capillary flow of water must also be considered in methods which involve the use of filter paper, since adsorption is not an instantaneous process, rate and extent being dependent upon temperature, viscosity, and other factors.

Methods

The experiments described in this paper were made with a modified Landsteiner and Pauli apparatus of the type (A) designed by Krueger, Ritter, and Smith (4). A slight modification of the electrodes was made by substituting zinc mercury

amalgam dipping into concentrated zinc sulfate solution as described by Olitsky (10). Preceding each use of the instrument the glass parts were boiled and rinsed with saline solution. The stop-cocks, lightly greased with sterile vaseline, were

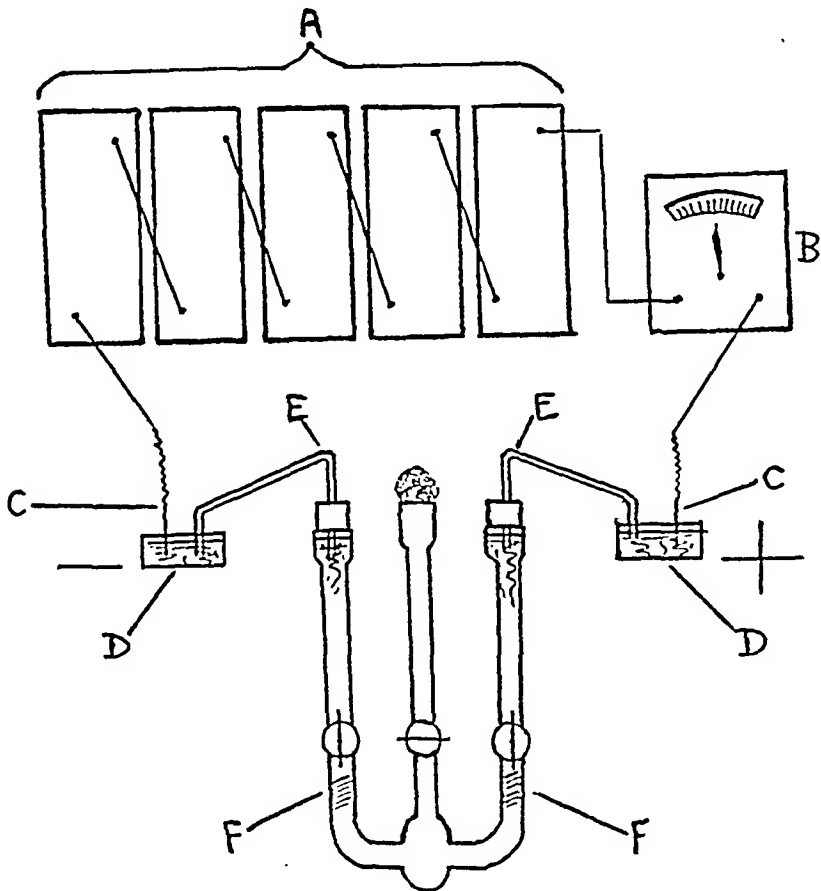


FIG. 1. Arrangement of the apparatus used in these experiments. (A) five dry batteries in series; (B) milliammeter; (C) zinc-mercury electrode; (D) saturated solution of zinc sulfate; (E) glass tubes containing 2 per cent salt agar; (F) cotton plugs to prevent diffusion.

then inserted and the bore of each was cleaned with a pledget of sterile cotton to remove excess vaseline. The side arms and bowl of the "U" tube were filled with diluted normal monkey serum. This was used instead of salt solution alone because it has been shown (11, 12) that the virus of yellow fever is soon inactivated

in serum-free fluids.¹ Sterile cotton pledgets, freed from air bubbles, were then pushed into the side arms below the stop-cock to prevent diffusion. The infectious material was placed in the central arm and after about 5 minutes approximately 2 cc. were allowed to flow down into the central bowl until the fluid, rising a millimeter or two in the side arms, made contact with the agar bridges. The bowl is so constructed as to form circular currents within itself, thus avoiding contamination of the side arm material with virus from the center.

In addition to the glass apparatus, the electrical circuit contained a milliammeter and five dry batteries each developing 22.5 volts. At the proper time the circuit was closed and allowed to remain so for from 3 to 5 hours, the time depending on the material under investigation. The current, direct and uninterrupted, the time, the hydrogen ion concentration, and the composition of the material under investigation varied in different experiments and each is described in detail in Tables I and II and in the individual discussions of the tests.

After passage of the electric current for the desired length of time, the circuit was opened, the stop-cocks were immediately closed, and the fluid in each arm was removed and injected intraperitoneally into a monkey (*Macacus rhesus*) as soon as possible. The material in the central arm served merely as an infectivity control, and was not exposed to the electric current. Monkeys that died were autopsied and the cause of death was determined by both gross and microscopical examination of the organs. Monkeys that survived were, in some instances, bled after a few weeks to obtain serum for complement fixation tests (14) and in all cases received an infective dose of virus to determine their continued susceptibility or possible immunization.

Experiments 1 to 8

In these tests the diluted normal monkey serum was clear and free from visible hemoglobin. It was freshly prepared each time from the serum of two or three monkeys and was diluted with sterile physiological salt solution or phosphate buffer solution as shown in Table I. The source of virus was clear serum, as free as possible from hemoglobin, taken from monkeys during their first febrile access due to yellow fever. The reaction in these eight tests ranged from pH 7.7 to 5.0. In Experiments 7 and 8, after mixing the normal serum and the pH 6.2 buffer solution the reaction remained pH 6.2, but it was necessary to adjust the reaction of the pH 5.0 mixture with a little N/20 hydrochloric acid. The neutral mixture (Experiment 6) was prepared as usual with salt solution and adjusted with a small amount of N/20 acid.

Experiments 9 and 10

In these tests, known infective mosquitoes which had not had a blood meal for at least two weeks were killed with tobacco smoke and ground up in diluted nor-

¹ This may have been the cause of failure in an earlier attempt to determine the charge of the yellow fever virus (13). In the experiments referred to, the virus was required to migrate into serum-free, 2 per cent salt agar.

mal monkey serum. After centrifugation, the emulsion was passed through a Berkefeld N filter and substituted for the virus-bearing serum under conditions otherwise exactly like those obtaining in Experiments 1 to 8. The details of these two mosquito experiments appear in Table I.

Experiments 11 and 12

These experiments were conducted in exactly the same manner as Experiments 1 to 10, except that the normal serum was diluted with distilled water instead of saline solution and contained enough erythrocytes to produce a deep red, translucent, somewhat turbid fluid, most of the red cells being hemolyzed. It was not possible to determine the reaction by the colorimetric method, because of the hemoglobin present, but it seems likely that it was well on the alkaline side of neutral.

RESULTS AND DISCUSSION

The data in Table I show that in clear, hemoglobin-free serum dilutions having slightly alkaline reactions (pH 7.4 to 7.7) active virus was demonstrated three times at the cathode (negative pole) and never definitely at the anode. Experiment 2 was unsatisfactory because the animal inoculated with material from the anode may have been one of the naturally refractory monkeys occasionally encountered. In Experiment 4 it is impossible to interpret the reactions of the monkeys as indicating either cataphoresis or anaphoresis. In Experiments 3, 5, 6, and 7 it seems that between pH 7.4 and pH 6.2 the virus first passes through its isoelectric range and then tends to reverse its direction of migration. It thus behaves like an amphoteric colloid. At a pH of 5.0 it was evidently inactivated (Experiment 8).

It is of interest to note that the mosquito virus migrated in the same direction as the serum virus. However, as has been pointed out, the migration of the virus may be due in large part to adsorption of some serum constituent which confers upon the virus uniform electrical properties regardless of origin.

That alterations in the composition of the suspending fluid may affect the migration of the virus is shown by Experiments 11 and 12, in which the virus migrated definitely to the anode (positive pole). It appears that the virus was influenced in some manner by the hemoglobin, by the red cells or their stroma, by some precipitate due to the addition of the water, by the low electrolyte content, or by some other unknown factor. It was observed, in conformity with the results of

TABLE I
Details and Results of Various Electrophoresis Experiments with Yellow Fever Virus

<div> <div>TABLE I</div> <div>Details and Results of Various Electrophoresis Experiments with Yellow Fever Virus</div> </div>									
Experiment	Mixture used to fill side arms of "U" tube	Reaction	Reactions of animal receiving material from:				Result		
			pH	Current ma.	Time hrs.	Positive arm (anode)			
						Negative arm (cathode)	Central arm (control)		
1	20 cc. normal serum plus 30 cc. salt solution	7.4	5	3	No fever. Complement fixation negative. Died of 2nd inoculation (yellow fever)	Fever. Complement fixation ++++. Immune to 2nd inoculation	Fever. Probably immune to 2nd inoculation	Virus migrated cathode	
2	15 cc. normal serum plus 30 cc. salt solution	7.2	5	3	No fever. Complement fixation negative. Immune to 2nd inoculation	No fever. Died 27th day with no fever. Immunity not tested	Fever. Complement fixation ++++. Immune to second inoculation		
3	17 cc. normal serum plus 30 cc. salt solution	7.3	5	4	No fever. Died of yellow fever from 2nd inoculation	Chronic fever. Complement fixation negative. Died of yellow fever from 2nd inoculation	Died of yellow fever	Inconclusive	
4	15 cc. normal serum plus 30 cc. salt solution	7.7	5	5	No fever. Survived 2nd inoculation with slight fever. (Immune?)	No fever. Survived 2nd inoculation with some fever. (Immune?)	Died of yellow fever	Virus failed to migrate (isoelect. range?)	
5	21 cc. normal serum plus 34 cc. salt solution	7.4	6	2	Fever. Not immune to 2nd inoculation	Fever. Died of yellow fever due to 2nd inoculation	Died of yellow fever	Inconclusive	
							Died of yellow fever	Virus failed to migrate	

6	10 cc. normal serum plus 35 cc. salt solution. Reaction adjusted with N/10 KOH	7.0	6	3.5	No fever. Died of yellow fever from 2nd inoculation	No fever. Died of yellow fever from 2nd inoculation	Fever. Immune to 2nd inoculation	Virus migrated to neither pole (isoelectric range)
7	15 cc. normal serum plus 30 cc. phosphate buffer solution	6.2	4.5, 2.5		Died of yellow fever	Fever. Complement fixation ++++. Immune to 2nd inoculation	Died of yellow fever	Virus migrated to both poles (acid)
8	17 cc. normal serum plus 18 cc. phosphate buffer solution. Reaction adjusted with N/10 KOH	5.0	4	3	No fever. Survived test dose with fever	No fever. Died of yellow fever from 2nd inoculation	No fever. Immune to 2nd inoculation	Inconclusive. Virus probably inactivated (acid)
9	20 cc. normal serum plus 10 cc. salt solution	7.5	5	4	Fever on 21st day. Died of yellow fever from 2nd inoculation	Fever. Complement fixation ++++. Immune to 2nd inoculation	Died of yellow fever	Virus migrated to cathode (mosquitoes)
10	22 cc. normal serum plus 25 cc. salt solution	7.7	5	4.5	No fever. Died of yellow fever from 2nd inoculation	Died of yellow fever	Died with intercurrent infection probably complicated with yellow fever	Virus migrated to cathode (mosquitoes)
11	25 cc. normal serum containing a good number of red cells plus 25 cc. distilled water	Not determined (color)	4	3	Died of yellow fever	Fever. Immune to 2nd inoculation	Died of yellow fever	Virus migrated to both poles (hemoglobin)
12	15 cc. normal serum containing many red cells plus 30 cc. distilled water	Not determined (color)	3.5, 3		Died of yellow fever	Fever. Died after 2nd inoculation, probably of yellow fever	Died of yellow fever	Virus migrated to anode (hemoglobin)

Michaelis (16) and others, that the hemoglobin migrated strongly to the anode, invading the agar bridge for a distance of about 15 mm. It is possible that the virus adsorbed hemoglobin and migrated as such, or that the virus was swept toward the anode by the rapidly moving hemoglobin in spite of its own slower motion toward the cathode.

These results agree, in many respects, with those of Hindle and Findlay. Using the filter paper method of Bedson and Bland (17) these workers concluded that in fluids having reactions between pH 5.2 and pH 7.0 the virus has a negative charge. They found the virus to be inactivated in fluids with a reaction more acid than pH 5.2. Thus it seems clear that the virus usually acts as though positively charged in alkaline fluids and as though negatively charged in acid fluids unless influenced by some factor such as hemoglobin or some other electrically active substance. The isoelectric point of the virus seems to be in the neighborhood of pH 7.0, possibly ranging from about 7.3 to 6.9. It is destroyed by acidities greater than pH 5.2.

Electrophoresis of Antibodies

We know, from the experiments of Field and Teague (18, 19), and many other workers, that various kinds of antibodies against bacteria and their products and against various proteins behave in many instances like colloids and migrate in an electrical field. In the literature at hand there is little to be found relating to the electrical properties of the protective bodies in antiviral sera. From the results of Olitsky and Long (6) and Olitsky, Rhoads, and Long (20), who electrically separated infective quantities of virus from non-infective tissues of vaccinia-recovered rabbits, and poliomyelitis-recovered monkeys, one may infer that the antibodies did not migrate or that they moved in a direction opposite to that of the viruses. In any case, the viruses, although neutralized by the antibodies, were certainly not inseparably combined with them. A rather loose physical combination is indicated.

Very little is known of the nature of the reaction by which yellow fever immune serum protects susceptible animals against infection. It seems not unlikely, however, that a physical combination, possibly based upon mutual neutralization of two oppositely charged colloids, may occur.

In order to test this hypothesis experiments were arranged to ascertain, if possible, the charge carried by the antibodies in yellow fever immune serum. These experiments were in all respects similar to Tests 1 to 10, except that serum of an immune monkey was substituted for the virus-bearing material in the central arm of the apparatus.

In most of these experiments, 1 hour after injection of material from each arm of the apparatus each monkey was inoculated with fresh, virus-bearing blood. When, in a given test, the monkey receiving material from the positive arm of the "U" tube gave no evidence of infection while the animal that received material from the negative arm died of yellow fever or had a severe febrile attack within the incubation period, it was assumed that protective bodies must have migrated to the anode (positive arm). This is in accord with the suggestion that the reaction between virus and antibody involves two oppositely charged colloids, since, as has been shown in the preceding pages, the virus migrates to the cathode as a rule.

As shown in Table II, in four tests the monkey receiving material from the cathodal arm died of yellow fever and in a fifth test had a severe attack of fever. In a sixth test the anodal monkey died of yellow fever. The latter experiment is vitiated, however, by a question which arose immediately after inoculation, as to the identity of the two animals. The balance of satisfactory evidence makes it seem that yellow fever antibodies do not migrate regularly to the cathode. Migration to the anode, on the other hand, occurred in three (or possibly four) of the six trials. Among the three trials in which the antibodies are not recorded as migrating to the anode is one (4) in which polarization of the electrodes occurred, with marked precipitation of the protein, difference in reaction of the fluid in the two side arms, heating of the apparatus, etc. The antibodies apparently did not migrate satisfactorily in either direction. In another trial (5) the anodal animal died, after three days, of another infection (tuberculosis) and all organs affected were riddled with tubercles. There was no evidence, gross or microscopic, that yellow fever was in any way involved. The antibodies may have migrated to the anode in this test. They certainly did not migrate to the cathode since the cathodal animal died of typical yellow fever. After reviewing all of the evidence, scanty though it is, it seems not unreasonable to conclude

ELECTROPHORESIS OF YELLOW FEVER VIRUS

TABLE II
Electrophoresis of Protective Bodies in Yellow Fever Immune Serum

Experiment No.	Mixture used to fill side arms of "U" tube	Reaction		Reactions of monkey receiving material from electrophoresis cell followed by inoculation with virus:				Remarks
		pH	Current	Time	Material from anode (positive arm)	Material from cathode (negative arm)	Material from central arm	
1	Normal serum 15 cc. plus saline solution 35 cc.	—	5	5.5	Died of yellow fever	Protected	Protected	Antibodies migrated to cathode (?). Error in animal numbers probable
2	Normal serum 15 cc. plus saline solution 30 cc.	7.4	5	5.5	Protected	Died of yellow fever	Protected	Antibodies migrated to anode
3	Normal serum with many erythrocytes 15 cc. plus distilled water 45 cc.	— (Color)	3	5	Had a slight fever for 36 hours on 6th day. Protected	Died of yellow fever	No control (same serum previously protected)	Antibodies migrated to anode (hemoglobin)
4	Normal serum 5 cc. plus saline solution 45 cc.	6.4 (+ arm) 7.6 (— arm)	5	5	Died of yellow fever	Fever and recovery	Same as above	Marked difference in pH of two arms with heavy precipitate. Probable polarization of electrodes. Inconclusive

5	Normal serum 17 cc. plus saline solution 33 cc plus 3/10 KOH	8 6	6	4	Died of intercurrent dis- ease (tuberculosis) on 4th day. No evi- dence of yellow fever grossly or microscop- ically	Died of yellow fever	Protected	Antibodies did not mi- grate to cathode. An- ode inconclusive
6	Normal serum 20 cc. plus saline solution 30 cc.	7 4	5 4 5	5 4 5	Protected Transitory fever on 9th and 10th days	Died of yellow fever	Protected	Antibodies migrated to anode

that the antibodies in yellow fever serum carry a negative charge under the conditions of these experiments and that the neutralization of virus by immune serum may involve an interaction between oppositely charged colloids.

CONCLUSIONS

1. When suspended in slightly alkaline (pH 7.4 to 7.8) saline dilutions of clear, hemoglobin-free normal monkey serum, the virus of yellow fever from infected monkeys and from infected, but blood-free, mosquitoes, usually acts as if it were possessed of a positive electrical charge.

2. The virus tends to assume a negative charge in fluids having a slightly acid reaction.

3. The isoelectric point of the virus seems to be in the neighborhood of pH 7.0, possibly ranging from pH 7.3 to pH 6.9.

4. Exposure to fluid having a reaction of pH 5.0 for 3 hours appeared to inactivate the virus.

5. In experiments in which the suspending fluid was prepared with normal serum diluted with distilled water and containing a good quantity of partly hemolyzed erythrocytes, the virus tended to migrate to the anode.

6. The protective bodies in yellow fever immune serum appear to carry a negative charge in slightly alkaline saline dilutions of serum.

The writer takes pleasure in acknowledging the assistance of Dr. Nelson C. Davis in conducting these experiments.

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STUDIES ON THE RELATION BETWEEN TUMOR SUSCEPTIBILITY AND HEREDITY

V. THE INFLUENCE OF HEREDITY UPON THE INCIDENCE OF LUNG TUMORS IN MICE

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The existence of constitutional types which differ in their susceptibility to disease has long been recognized. Of recent years the subject has received special emphasis in the numerous investigations which have been carried on to determine whether such types are inherited. The differences may manifest themselves in the degree of resistance to bacterial disease, or may be apparent in the frequency of pathological conditions of non-bacterial origin. In either case the demonstration of the inheritance of distinct types may aid in isolating them and also contribute to the analysis of the phenomena of resistance and susceptibility.

A considerable number of data have been collected in our laboratory concerning the incidence of lung tumors in mice. It has already been shown (1) that strains of mice exist which differ significantly from each other in regard to the incidence of spontaneous lung tumors which occur in them. It has also been demonstrated that the same strains differ in the same general way in their liability to induced lung tumors. That the latter differences are inherited has been shown by appropriate crosses (2). The exact relation between induced and spontaneous tumors has not been determined. It is possible that they are identical as far as the nature of the susceptibility is concerned, but it is important to know whether data on spontaneous tumors in an experiment involving a cross between strains would parallel those yielded by induced tumors. Evidence bearing on this point is now at hand.

A cross was made between a low tumor strain and one which proved to be considerably higher. Only one male representing the high strain was used (No. 7001 in Fig. 1). It came from a stock that had been pen inked for several years.

Cross between ♂ No. 7001 and 6 ♀♀ from Strain 1194

♂ parent		♀ parents	F ₁ indicated by age in months at time of death.	
♂ No. 7001 9 mo.	×	♀ No. 7001.5 13 mo.	♂ 7 N.A. ♀ 8 ♀ 8 ♀ 9 M.met. ♀ 11 ♀ 13 ♂ 18 N.A. ♂ 19 ♀ 22 M. ♀ 26 N.A.	♂ 28 P.
"	×	♀ No. 7001.46 17 mo.	♀ 5 ♀ 6 ♀ 8 M. ♀ 8 M.met. ♀ 11 ♀ 16 ♀ 23 ♂ 31 ♂ Living	♂ 27 P. ♂ 28 P. ♂ 32 P.
"	×	♀ No. 7001.13 12 mo.	♀ 5 ♀ 7 M. ♀ 8 M. ♀ 8 ♀ 10 ♀ 11 ♂ 16 ♂ 24 ♂ 30	♂ 22 P.met. ♂ 26 P.
"	×	♀ No. 7001.16 13 mo.	♂ 6 ♀ 7 M.met. ♀ 7 M.met. ♀ 7 ♀ 8 M.met. ♀ 8 M.met.	♀ 11 P. ♂ 26 P. ♂ 33 P. ♂ 35 P.
"	×	♀ No. 7001.2 12 mo.	♀ 10 M.met. ♀ 18 M. ♂ 21 ♂ 31	♂ 27 P.
"	×	♀ No. 7001.6 15 mo.	♂ 7 ♀ 7 ♀ 18 M.met. ♀ 22 M.met.	

Among 45 mice over 6 months old 11 (24.4%) have lung tumors
 " 24 " " 12 " " 10 (41.6%) " " "
 " 20 " " 18 " " 10 (50.0%) " " "

FIG. 2. M. = mammary tumor; met. = metastatic; N. A. = not autopsied.
 P. = pulmonary tumor.

Although it died at 9 months without having a tumor, mated with a sib which had a lung tumor at 12 months of age, it produced fourteen young, two, possibly three of which had lung tumors, that is to say, lung tumors appeared in the first inbred generation. These descendants were backcrossed to the male parent and inbred in various ways. Among 89 offspring belonging to several generations, that lived more than 6 months, 39.3 per cent had lung tumors; and among twenty-five mice over 18 months of age, 52.2 per cent had tumors; and among mice over 12 months, 64.0 per cent were tumorous. Other branches of this same stock (D) from which the male was selected have given a rate of 34 per cent among 222 mice over 6 months of age, so that it is apparent we are dealing with a strain fairly rich in pulmonary tumors. Male No. 7001 was crossed also with six females from Strain 1194. This strain has a lung tumor incidence of 6.7 per cent in mice over 1 year old, and the earliest age at which tumors appear is 18 months. The female parents and their offspring are charted in Fig. 2. The female parents died at various ages between 12 and 17 months—that is, below the usual tumor age in this strain. None had tumors of any sort. Every parent had amongst its immediate offspring one or more individuals with lung tumors, except No. 7001.6, which had only two daughters living well into tumor age, too small a number to be a test of her genetic constitution. This suggests, though it does not prove, that susceptibility may be dominant—or rather semidominant, since age and other influences must prevent the somatic expression of the tumor character in many individuals which genetically are tumor mice. There were in all forty-five F_1 mice that lived more than 6 months. Their lung tumor rate was 24.4 per cent. The twenty-four F_1 that lived more than a year had a rate of 41.6 per cent, and twenty mice that lived more than 18 months had a rate of 50 per cent. The numbers involved are small and these rates are not significantly different from those given by the descendants of male No. 7001 when inbred with a sib. The 14 mammary tumors which also occurred in this generation need not be considered here.

Males from the first filial generation were backcrossed to females of both parental strains. Forty-two backcross mothers were selected from Strain D. Of thirty-one that lived more than 6 months, 39 per cent had lung tumors—a rate like that of the inbred stock. From the low tumor stock, thirty-nine mice were used as the female parents. Only three lived more than 18 months and none more than 23 months. None had tumors.

The offspring from these crosses are charted in Fig. 3, which gives the distribution according to age at death of all mice that lived more than 6 months, except sixteen upon which no autopsy was obtained. Males and females of each group are represented separately. Among the males there were two doubtful cases of lung tumor, one at 18 and the other at 25 months. Both occurred in the backcross from the low tumor strain and in the totals were treated as tumor mice. Among the females the only complications as to diagnosis arose in connection with indi-

viduals that had tumors in the mammary gland. In such cases it was sometimes difficult to distinguish between primary lung tumors and metastases from mammary tumors. Among 331 females over 6 months of age 62 or 18.7 per cent had mammary tumors and twenty or 6.0 per cent had tumors which were diagnosed as primary both in the mammae and in the lung. In a total population of 622

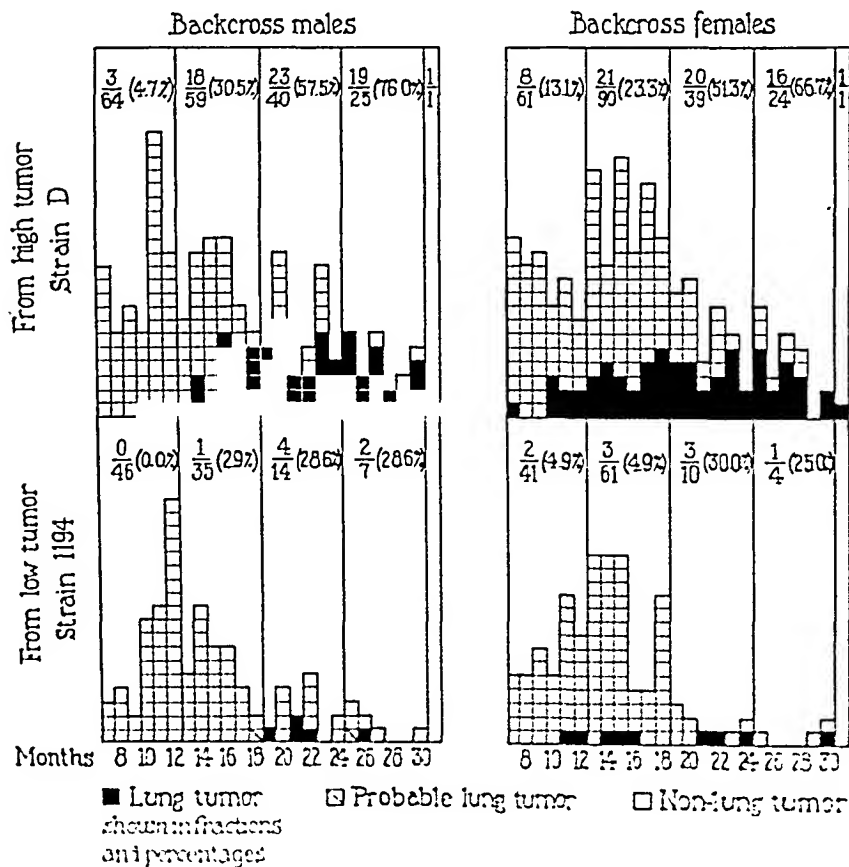


FIG. 5

backcross individuals there were two mice exhibiting epithelioma of the jaw, one being a female which had also a mammary tumor. There were four mice with sarcoma. Three of these sarcomas were subcutaneous. One of the mice died in a mouse which had a lung tumor, and another in an individual which had both primary and lung tumors. The fourth sarcoma probably had its primary site in the uterus with extensive metastases to the lungs, liver, kidney and other organs.

No tumors except those in the lung are indicated on the chart. The individuals are grouped in age periods of 6 months.

If comparisons are made between corresponding age groups of the same sex (Table I) it is found that in every case the percentage of lung tumors is greater in the backcross from the high tumor strain than in that from the low strain. In the first age period, that between 7 and 12 months, in which the number of tumors is small, the difference in the percentages of the two backcross groups is, for the males, only 2.6

TABLE I

Comparison of the Backcross Groups—Males and Females Considered Separately

Sex	Age	Backcross from Strain D				Backcross from Strain 1194					
		Total mice	No. of mice with tumor	Per cent of mice with tumor	P.E. of per cent	Total mice	No. of mice with tumor	Per cent of mice with tumor	P.E. of per cent	Difference of percentages	P.E. of difference
♂ ♂	mos.										
	7-12	64	3	4.7 ± 1.8		46	0	0.0 ± 0.0		4.7 ± 1.8	2.6
	13-18	59	18	30.5 ± 4.0		35	1	2.9 ± 1.9		27.7 ± 4.5	6.2
	19-24	40	23	57.5 ± 5.3		14	4	28.6 ± 8.1		28.9 ± 9.7	2.9
	25-31	26	20	76.9 ± 5.6		7	2	28.6 ± 11.5		48.4 ± 12.8	3.8
Total.		189	64	33.9 ± 2.3		102	7	6.9 ± 1.7		27.0 ± 2.9	9.4
♀ ♀	7-12	61	8	13.1 ± 2.9		41	2	4.9 ± 2.3		8.3 ± 3.7	2.2
	13-18	90	21	23.3 ± 3.0		61	3	4.9 ± 1.9		18.4 ± 3.5	5.2
	19-24	39	20	51.3 ± 5.4		10	3	30.0 ± 9.8		21.3 ± 11.2	1.9
	25-31	25	17	68.0 ± 6.3		4	1	25.0 ± 14.6		43.0 ± 15.9	2.7
Total.		215	66	30.7 ± 2.1		116	9	7.8 ± 1.7		22.9 ± 2.7	8.5

times its probable error—that is, merely possibly significant—and for the females it is only 2.2 times its probable error. For the period between 13 and 18 months the differences in the two backcrosses are clearly significant for both sexes. In the two later age periods the total numbers involved, especially in the backcross from the low tumor strain, were very small.

To compare the groups as a whole a method can be employed which has been described by Karl Pearson and J. F. Tocher (3). It was designed to give a more accurate comparison than that previously

possible between the death-rates of two groups of individuals in which the age distribution differed and in which death might be due to diseases which are a function of age. By this method the death rate is corrected by reduction to a "standard population" and a constant (Q) is found which expresses the ratio of the difference of corrected death-rates to the standard deviation of that difference. Since Q divided by 0.67449 is equal to the ratio of the difference of the percentages to the probable error of the difference, this constant may be used to make comparisons similar to those of the preceding paragraph. To test further whether the two systems of frequency are random samples of the same population the general χ^2 test of partial contingency also may be applied. Considering the males first we find in our present data that the crude tumor rate of the total population of males from the backcross to the high tumor strain is 33.9 ± 2.3 per cent and from the low strain 6.9 ± 1.7 per cent with a difference of 27.0 ± 2.9 per cent or 9.4 times its probable error. By applying the method of Pearson and Tocher to the groups of individuals in Table I the value of Q^2 is found to be 21.9054 and of Q , 4.683. Here Q divided by 0.67449 would be 6.9. While this figure is smaller than that obtained by the first method there is no doubt that the two populations are significantly different. In the partial contingency test the value of χ^2 proves to be 20.2484. Referring to Elderton's Table (4) we find by interpolation that $P = 0.00045$ for $n' =$ five groups. Again the chances are tremendously against the probability that random sampling would account for the divergence found in the two populations. Similarly for the females, the crude tumor rates are 30.7 ± 2.1 per cent and 7.8 ± 1.7 per cent for the backcrosses to the high and low strains respectively. The difference is 22.9 ± 2.7 or 8.5 times its probable error. From the data in Table I, the value of Q^2 is found to be 15.2602 and the difference of the two groups according to their "corrected" tumor rates would be equivalent to 5.8 times the probable error. Furthermore, χ^2 is 14.1897 and P is 0.0068. Measured by these tests the backcross groups, both male and female, derived from the high tumor strain gave significantly higher tumor rates than did the corresponding backcross groups from the low tumor strain.

Thus far we have dealt with the sexes separately. In some of our previous data there have been indications that males are somewhat

more susceptible to lung tumors than females. A detailed analysis, however, of strictly comparable groups has not shown a significant difference. Despite this fact the possibility that sex may have a slight influence has not been precluded and a further investigation dealing with more critical evidence is under way. It may be of interest to examine the present data from this point of view. From Table II it will be seen that in the backcross from Strain D in three age periods the males have a higher tumor incidence, though none of them significantly so, and the total incidence is 3.2 per cent higher than that of the females. This figure is scarcely greater than its probable error (± 3.1 per cent). In the backcross from Strain 1194 in three age periods

TABLE II
Comparison of Males with Females in the Two Backcross Groups

Age	Backcross from Strain D			Backcross from Strain 1194		
	Difference of tumor percent-ages	P.E. of difference	$\frac{\text{Difference}}{\text{P.E.}}$	Difference of tumor percent-ages	P.E. of difference	$\frac{\text{Difference}}{\text{P.E.}}$
<i>mos.</i>						
7-12	-8.4 \pm 3.4		2.5	-4.9 \pm 2.3		2.1
13-18	7.2 \pm 5.0		1.4	-2.1 \pm 2.7		0.8
19-24	6.2 \pm 7.5		0.9	-1.4 \pm 12.7		0.1
25-31	8.9 \pm 8.4		1.1	3.6 \pm 18.6		0.2
Total.....	3.2 \pm 3.1		1.0	0.9 \pm 2.4		0.4

. - sign indicates that the female showed the higher rate.

it is the females that have a higher tumor incidence and the total rate for the females is just slightly larger (0.9 ± 2.4 per cent) than that of the males. However, a greater difference between the sexes is brought out by the Pearson and Tocher method. Comparing the males in the backcross from Strain D with the females we find Q^2 to be 4.5306 and Q divided by 0.67449 to be 3.2. However, further calculation shows χ^2 to be 4.5063 and P to be 0.346. That is, according to the latter test the chances are about 35 in 100 that random sampling would account for the divergence noted in the two systems. The higher rate of the males cannot, therefore, be regarded as significant. In the other backcross groups (from Strain 1194) $Q^2 = 2.5792$ and Q divided

by 0.67449 is 2.4. For these data, χ^2 proves to be 2.5386 and P is 0.639. The higher rate of the females in this case is not significant.

More extensive data as to the effect of sex upon lung tumor incidence are being compiled. In the meantime, since no influence has been demonstrated, it may be permissible to class males and females together and compare the total populations in the two backcrosses. The combined data are listed in Table III and it will be noted that at every age period the differences are statistically significant. For a total of 404 mice obtained from backcrossing to the high tumor strain, the lung tumor incidence was 32.2 ± 1.6 while 218 individuals from the low tumor strain had a tumor rate of 7.3 ± 1.2 . This gives a difference of 24.8 ± 2.0 per cent, over twelve times its probable error.

TABLE III

Comparison of the Backcross Groups—Males and Females Considered Together

Age	Backcross from Strain D				Backcross from Strain 1194				Difference of percentages	P.E. of difference	Difference P.E.
	Total mice	No. of mice with tumor	Per cent of mice with tumor	P.E. of per cent	Total mice	No. of mice with tumor	Per cent of mice with tumor	P.E. of per cent			
<i>mos.</i>											
7-12	125	11	8.8 \pm 1.7		87	2	2.3 \pm 1.1		6.5 \pm 2.0		3.2
13-18	149	39	26.2 \pm 2.4		96	4	4.2 \pm 1.4		22.0 \pm 2.8		7.9
19-24	79	43	54.4 \pm 3.8		24	7	29.2 \pm 6.3		30.3 \pm 7.3		3.5
25-31	51	37	72.5 \pm 4.3		11	3	27.3 \pm 9.1		45.3 \pm 10.0		4.5
Total	404	130	32.2 \pm 1.6		218	16	7.3 \pm 1.2		24.8 \pm 2.0		12.6

If the method of Pearson and Tocher is again applied, the value of Q proves to be 6.009 from which we may deduce that the difference in the rates of the groups in question would be equivalent to 8.9 times the probable error. χ^2 is 33.3458. In Elderton's Table for $\chi^2 = 30$ we find P to be 0.000005. There is no question of the significance of the figure obtained here.

Table IV is appended in order to give another grouping of the same data which may be more convenient for comparison with previously published records of other strains. Here the differences in the tumor rates of the contrasted populations for mice over 6 months, 12 months and 18 months of age are undoubtedly significant.

Conclusions as to the number of genetic factors cannot be drawn with certainty. Several interpretations are possible. Although the female parents and the backcross mothers from Strain 1194 were without tumors, there is a possibility that growths might have developed if the females had lived longer. It is not yet clear just what the character is that is inherited. It may be that it is a type of constitution which has a tendency to react in a certain percentage of individuals in the production of neoplastic disease. According to this theory, tumor mice would not all be alike; there might be a gene corresponding to each of the various tumor percentages. This method of analysis has sometimes been applied to the tumor problem. It naturally results

TABLE IV
Comparison of the Backcross Groups—Males and Females Considered Together

Age	Backcross from Strain D				Backcross from Strain 1194						
	Total mice	No. of mice with tumor	Per cent of mice with tumor	P.E. of per cent	Total mice	No. of mice with tumor	Per cent of mice with tumor	P.E. of per cent	Difference of percentages	P.E. of difference	Difference P.E.
Over 6 mos.	404	130	32.2 ± 1.6		218	16	7.3 ± 1.2		24.8 ± 2.0		12.6
Over 12 mos.	279	119	42.7 ± 2.0		131	14	10.7 ± 1.8		32.0 ± 2.7		11.8
Over 18 mos.	130	80	61.5 ± 2.9		35	10	28.6 ± 5.2		33.0 ± 5.9		5.6

in quite different conclusions from those obtained if either a single pair of factors with wide somatic fluctuations or multiple factors are considered as the heritable units.

From the pathological standpoint, differences in susceptibility have often been remarked. There is evidently an extensive series of conditions from highly susceptible to highly insusceptible. Animals which are classed as "tumor mice" may have nodules of very different size and malignancy. Individuals from the low tumor strain which are classed as "positive" may have but one nodule a fraction of a millimeter in diameter, while in mice of the high tumor strain all lobes of the lung may be peppered with nodules of various sizes. Occasionally the tumor mass may occupy the entire lobe. Histologi-

cally, lung tumors may be composed of alveolar, terminal bronchial or bronchial epithelial cells, occurring separately, in the same animal, or even in the same nodule. Sometimes the growths appear benign, but may show infiltration and metastasis to other organs. Nothing is known as to their rate of growth. A classification which would be satisfactory for genetic purposes has not been worked out. How much of this variation is controlled by heredity is not clear.

DISCUSSION

The results presented here may perhaps be more illuminating if viewed in connection with some of the observations previously made in this laboratory upon the inheritance of lung tumors in mice. As a fundamental consideration for this study, it must be remembered that, when mice are crossed, parents both of which are tumorous may produce offspring which develop tumors and others which do not though they live well into the tumor age; likewise, parents which live to an advanced age without exhibiting growths in the lung may number among their progeny both tumor and tumor-free mice. This phenomenon could be explained either by somatic variability or on the basis of several genetic factors (5).

Regardless of the number of hereditary units the fact of the somatic variability of the tumor character should be obvious although it has apparently been disregarded by many authors. An individual that dies without producing a tumor is not thereby proved to be "non-tumor" genotypically. Tumor incidence varies with age, with sex and with environmental conditions, of which a few are known and probably a larger number unknown. It is plain that an individual genetically susceptible (carrying the gene or genes for tumor) might never actually develop a growth if the requisite secondary influences were non-operative. This impossibility of classification by inspection is one of the great obstacles to the genetic study of cancer. Since accurate identification of the non-tumor individual is impossible it is customary to make comparisons between groups of individuals on the assumption that the unknown, secondary, non-hereditary factors operate equally in each and then to make corrections for age, sex and whatever environmental influences can be checked.

During the course of our investigations a number of such comparisons

have been made and some of the more important items are outlined in Fig. 1. A necessary preliminary to the work included an extensive survey of the tumor incidence and tumor age of various strains of mice. Two strains were discovered (1), one derived from Bagg albinos and the other an agouti strain, No. 1194, which have been demonstrated to differ significantly in their lung tumor rates. Among mice of all ages over 12 months Strain 1194 has 6.7 ± 1.2 per cent, and the Bagg albino has 37.0 ± 2.8 per cent of growths in the lung. The numbers are sufficient for the differences to be mathematically significant, thus showing that the hereditary tendencies of the two strains differ in respect to tumor incidence in the lung. When individuals from these strains are housed together in the same cages they have the tumor rates characteristic of their respective families and show no evidence of contamination (unpublished data). Since it is known that tumors can be induced by chronic irritation, it becomes a matter of importance to note whether or not these two types of constitution hereditarily maintained separate lines of descent react in the same manner to a carcinogenic agent. Groups of mice from the albinos and the agoutis have been tested by tar painting, the tar applications being made in the customary way in the interscapular area of the skin. The result of the experiment showed that tumors in the skin were produced in both groups at about the same rate and in the same percentage (64.3 ± 9.0 and 63.2 ± 8.7 per cent respectively). The numbers of mice in the experiment were small, but there was no indication that the difference in susceptibility which has been observed in regard to lung tissue applies also to the skin. From this it seemed that tumor susceptibilities might be specific. A further investigation of this possibility is being made at the present time. It was evident that instead of testing by the induction of tumors in the skin, a proper test of these two mouse strains would be one which would affect the tissue upon which the original observations were made. In the first experiment the albino strain had given a high percentage of lung tumors but it was not clear whether they were spontaneous or had been induced by the treatment. A more precise test was subsequently made possible through the discovery by Murphy and Sturm (7) that primary tumors could be induced in the lungs of mice by cutaneous tarring provided the area of application were varied. This modified method was therefore

applied to the strains in question. When the animals were about 13 months of age they were killed and it was found that the tumor rate in each strain had increased. The low strain gave a tumor incidence after tarring of 22.4 ± 4.0 per cent while that of the high tumor strain had gone up to 85.4 ± 3.4 per cent. It is to be noted that in the groups upon which the rate of spontaneous tumors was calculated there were no lung tumors at all in mice of either strain under 15 months of age. The difference between the two strains, shown by the incidence of tar-induced tumors, was twice as great as that in the spontaneous lung tumor rates. In an attempt to discover whether these hereditary qualities could be followed when the strains were crossed certain individuals from the two strains had been mated before they were subjected to tarring. When the resulting F_1 mice were painted, the tumor rate remained high (78.6 ± 5.2 per cent). The F_1 were then backcrossed to the two original stocks, and the offspring were tarred. The backcross from the Bagg stock remained high (81.1 ± 4.3 per cent), whereas in the backcross to the low strain the rate dropped to about half (39.5 ± 5.3 per cent).

As we have seen, these results have been duplicated by an experiment with spontaneous tumors. A male (No. 7001), from a strain in which lung tumors are frequent, was crossed with females from the same low tumor strain as that used in the preceding experiment, and their F_1 , when backcrossed to the parental stocks, gave high (32.2 ± 1.6 per cent) or low (7.3 ± 1.2 per cent) tumor rates according to which parental strain was used. The familial influence is again evident.

SUMMARY

A male mouse from a strain with a high incidence of spontaneous lung tumors was crossed with several females derived from a low tumor strain. The first generation of offspring were then backcrossed to individuals of the original strains. The resulting two groups of offspring differed significantly in the incidence of spontaneous tumors of the lung.

These facts are discussed in relation to others previously discovered.

It seems clear from the evidence presented that there are among mice constitutional types which differ in incidence of tumors of the

lung and that the differences are inherited. The number of genetic factors involved has not been determined. No influence of sex was apparent. The possibility of there being genetic factors which affect tumor age will be dealt with later.

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HISTOLOGICAL STUDIES OF THE BONE MARROW IN FASTED AND POLYNEURITIC PIGEONS

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PLATES 55 AND 56

(Received for publication, July 11, 1931)

In previous communications,¹ one of us has reported a series of studies on rice disease in pigeons and the effect of vitamin B, lactose, magnesium sulfate, and mineral oil on the anemia, weight loss, and bacteremia. In further study of this problem it was necessary to examine the histological changes in the bone marrow in rice disease and compare them with that in starvation and pure vitamin B deficiency. The present report concerns the results of this investigation.

Method

Adult healthy pigeons, which weighed about 350 gm., were used. Six were selected at random, fed on a normal diet of whole mixed grain and grit, and employed as controls. Eleven were deprived of all food except water until they had lost 40 to 50 per cent of the original body weight. Eight were allowed to eat polished rice until they had lost a similar weight. No attempt was made to force-feed these animals. Seven were fed a synthetic diet deficient only in vitamin B until definite polyneuritis developed. These animals were fed 12 to 18 gm. of the diet daily and watched to avoid regurgitation. This diet was prepared as follows:

Commercial casein was rendered vitamin B free by extraction three times with hot 80 per cent alcohol and mixed into a dough with the other constituents.

	per cent
Extracted casein	18
Starch	55
Crisco	20
Salt mixture.....	5
Cod liver oil.....	2

A 2 weeks supply was made at one time and kept in the ice box. When each animal developed a satisfactory experimental condition, it was sacrificed by di-

¹ Barlow, O. W., *Am. J. Physiol.*, 1930, 93, 161.

BONE MARROW IN POLYNEURITIC PIGEONS

vision of the neck and allowed to exsanguinate. The entire bone marrow from both radii was fixed in Zenker's solution, embedded in paraffin, and cut in longitudinal section so that the full length could be examined. The marrow from other bones was not examined. Sections were also prepared of the liver, spleen, kidneys, heart, and lungs. All sections were stained with hematoxylin and eosin and some with the Prussian blue and ammonium sulfide reactions for iron. In a few instances smears of the marrow were stained with eosin and methylene blue.

RESULTS

The general state of the fasting and rice disease birds at the time of sacrifice is shown in Tables I and II. An average of the rice disease

TABLE I
Rice Series

No.	Per cent of normal weight	R.B.C.	Hemoglobin	Bacterial count total
			<i>per cent</i>	
B1	53.7	2,152,000		
B2	65.3	3,008,000	54	82,000
B5	53	2,320,000	68	67,000
B12	62.5	2,832,000	57	110,000
C1	60	2,888,000	62.5	108,000
C3	63	2,304,000	67	68,000
C5	64.8	2,784,000	60	72,622
C10	63	2,788,000	66	13,920
C7	61.5	2,808,000	65	58,790
C13	66	3,048,000	66	79,000
Small	64	2,888,000	70	40,000
Median—11...	63	2,808,000	73	35,554
			66	68,000

birds shows 63 per cent of normal body weight; 2,808,000 red cells per c.mm., 69 per cent of normal; 66 per cent hemoglobin; and a bacterial count of 68,000. In the fasted group the weight was 60.3 per cent of normal; the red cell count 2,656,000, 65 per cent of theoretical; the hemoglobin 68 per cent; and the bacterial count 10,360. The degree of anemia and weight loss in these two groups is about equal.

In the fasted series the changes are similar to those described by Doan, Cunningham, and Sabin.² There is a fat replacement of the

² Doan, C. A., Cunningham, R. S., and Sabin, F. R., *Carnegie Institution of Washington, Pub. No. 83, Contributions to Embryology*, 1925, 16, 163.

marrow and marked decrease in hematopoietic elements. The myelocytic islands are for the most part absent except in the peripheral zones (Fig. 1, *b*). Erythrogenesis is almost in abeyance. In some animals (13, 20, 21, and 25) there is an increase in apparent vascularity and slight evidence of formation of red cells similar to the picture described by Doan, Cunningham, and Sabin as early regeneration (Fig. 2). With no refeeding which would produce this change, the conclusion is that it represents a failure of complete aplasia and not a regeneration after aplasia.

In the rice disease birds the histological changes in the bone marrow are strictly comparable to those of the fasted animals (Fig. 1, *c*). Again,

TABLE II
Fasting Series

No.	Per cent of normal weight	R.B.C.	Hemoglobin	Bacterial count total
			<i>per cent</i>	
A	54	1,984,000	62	12,864
B	64.6	3,136,000	72	9,408
C	61.6	2,512,000	65	12,560
D	52	1,760,000	62	10,560
E	68	3,088,000	76	10,190
F	53.2	2,032,000	59	10,160
G	55	1,920,000	68	10,360
H	59	3,256,000		
C8	63.4	2,800,000	74	14,000
C11	69	3,208,000	78	9,728
Median—10..	60.3	2,656,000	68	10,360

in Animals 9 and 12 there is a similar type of slight erythrogenesis. Thus on the basis of histological examination the conclusion must be, that the anemia of rice disease, in which the animals are not force-fed, is essentially a starvation anemia and not at all, or only indirectly, related to the absence of vitamin B in polished rice.

In the group of pigeons fed a synthetic diet deficient only in vitamin B, the type of change is entirely different. Hematopoiesis of both the granular and red cell series is abundant and shows all stages, but many of the adult and immature cells show degenerative changes (Fig. 1, *d*). The nuclei of myelocytes and megaloblasts show karyorrhexis, pyk-

BONE MARROW IN POLYNEURITIC PIGEONS

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nosis, and karyolysis and the cytoplasm of these cells is granular and vacuolated. (Fig. 3.) The cytoplasm of some erythrocytes stains a light bluish green and the cell walls are indistinct. This change is disposed throughout the marrow in irregular islands not over 500 microns in diameter. There is also an edema of the walls of the arterioles and venules with swelling and early proliferation of the endothelium of these vessels. It is not possible at this time to explain these changes on a definite etiological basis, but it is possible that the degenerative changes are secondary to the vascular disease. In the seven animals which we have examined, there is no alteration in erythrocytogenesis and no change similar to that observed in the rice disease birds.

Both the polished rice and synthetic diets are deficient in vitamin B, but the rice is also deficient in many other factors. On the basis of our histological observations, the changes in the bone marrow are unrelated and hence we believe that the anemia of rice disease, cannot be accepted as the result of vitamin B deficiency alone.

Sections of the liver, spleen, lungs, and heart show no pathological change other than a variable deposit of an iron-containing pigment in the endothelial cells of the hepatic and splenic sinusoids. We have been unable to distinguish the fasted and rice disease birds on the basis of the quantity or type of this pigmentation.

CONCLUSIONS

1. The histological changes of the bone marrow in fasted and rice disease pigeons are essentially the same.
2. The histological changes of the bone marrow in pure vitamin B deficiency consist of degeneration and edema and slight endothelial proliferation of the small vascular channels, but with active hemato-poiesis.
3. The anemia of rice disease in pigeons is in large part a starvation anemia and not directly related to vitamin B deficiency.

We wish to thank Doctor C. A. Doan for aid in this investigation.

EXPLANATION OF PLATES

PLATE 55

FIG. 1. (*a*) normal bone marrow, (*b*) bone marrow from fasted pigeon, (*c*) bone marrow from rice disease pigeon, (*d*) bone marrow from polyneuritis pigeon. $\times 90$.

PLATE 56

FIG. 2. From fasted pigeon. Note the apparent increase of vascular channels with many immature red cells within the lumina. $\times 325$.

FIG. 3. Island of hematopoietic tissue from pigeon fed a diet deficient in vitamin B. Note the swelling, granularity, and nuclear degeneration in the immature blood cells with edema of the articular wall. $\times 560$.

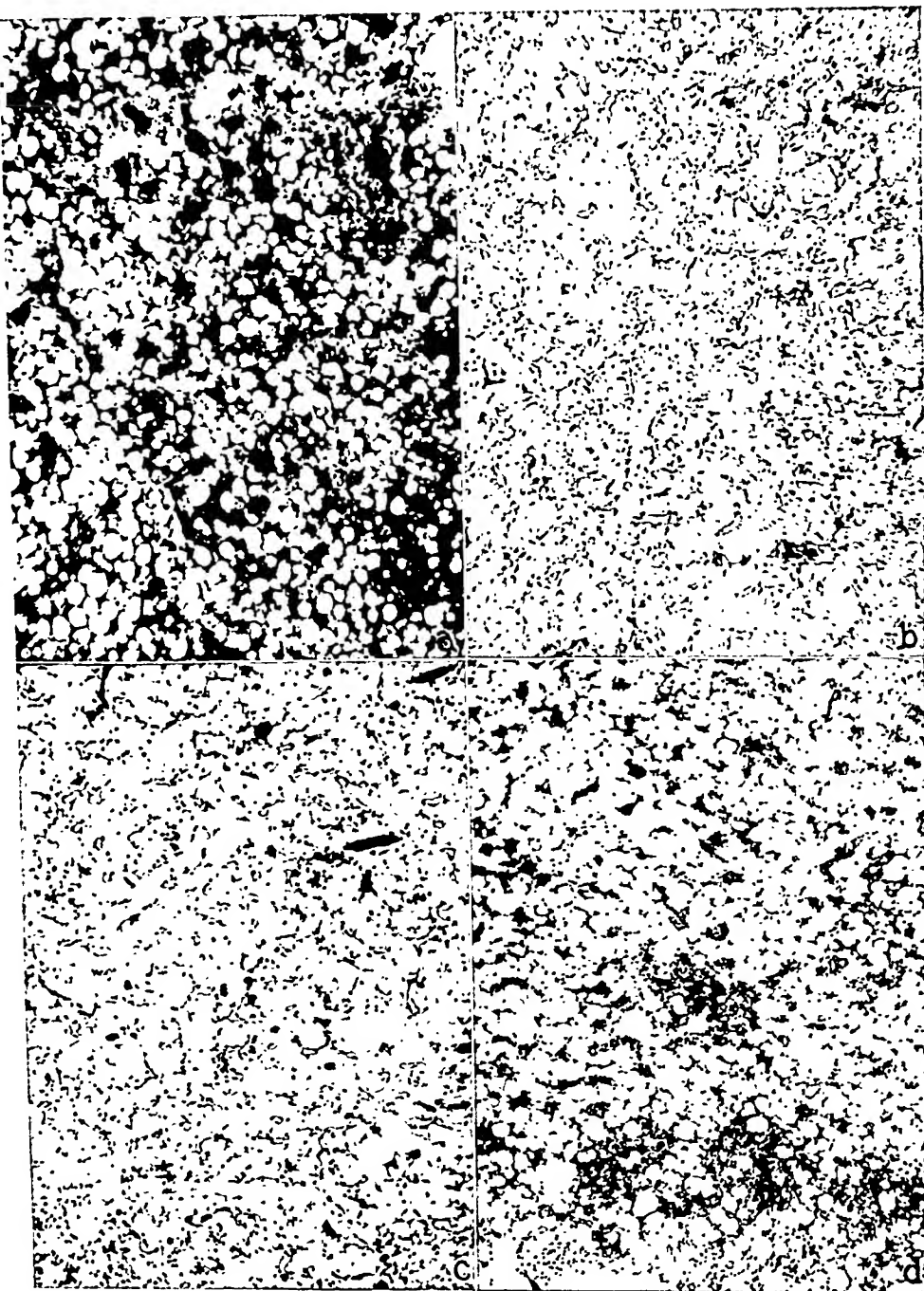


FIG. 1

(Moore and Barlow: Bone marrow in polynuritic pigeons)

THE IMMEDIATE EFFECTS OF UNILATERAL NEPHRECTOMY ON OPEN GLOMERULI AND URINE OUTPUT

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(Received for publication, July 11, 1931)

The phenomenon of oliguria or anuria following operation or manipulation of the urinary tract is well recognized. Few have attempted a definite explanation except on the basis of a ureterorenal or renorenal reflex. Such a hypothesis as this assumes a nervous pathway but does not adequately explain the fundamental changes in the kidney or kidneys. Bieter¹ has recently studied the effects of splanchnic section and stimulation on the blood-containing glomeruli of the frog. With the technique of direct observation he found that the splanchnics were concerned in the control of the glomerular circulation and felt that such a control explains reflex anuria. The Janus green B method of Hayman and Starr.² offers another method of approach to this problem and the present paper reports a study of this type.

Method

Two groups of rabbits were studied. In one group, unilateral nephrectomy was done and the animal allowed to recover for 2 hours when the opposite kidney was injected. In the other group, the animal was placed under sodium barbital anesthesia, the right kidney removed and a constant record of blood pressure kept for the 2 hours, when the left kidney was injected. In all experimental animals the right kidney was removed by an extraperitoneal lumbar operation and injected supravivally as described by Moore and Lukianoff.³ The bladder was emptied by catheter at the beginning and by catheter and inspection of the organ at the end of each experiment. Sodium barbital for anesthesia was given intravenously. The blood pressure was recorded from the carotid artery with a mercury manometer and a Becker kymograph. The phenolsulfonephthalein excretion was determined for a period of 1 hour after intravenous injection. Intravital injections and counts of both kidneys were carried out as described by Moore and Lukianoff.³

¹ Bieter, R. N., *Am. J. Physiol.*, 1930, **91**, 436; *Proc. Soc. Exp. Biol. and Med.*, 1928-29, **26**, 792.

² Hayman, J. M., Jr., and Starr, I., *J. Exp. Med.*, 1925, **42**, 641.

³ Moore, R. A., and Lukianoff, G., *J. Exp. Med.*, 1929, **50**, 227.

UNILATERAL NEPHRECTOMY

TABLE I
Unilateral Nephrectomies

Rabbit No.	Weight kg.	Urine volume			P.S.P. percent	Anesthesia	Average blood pressure		Weight cortex gm.	Weight sample gm.	Count on sample	Estimate entire kidney	Per cent open glomeruli
		1st hour cc.	2nd hour cc.	Total cc.			mm. Hg	mm. Hg					
17	2.3	75		0	0	Ether			S-5.98 I-5.34	0.58 0.58	18,441 4,779	196,844 43,991	23.3
10	1.5	75		16	70	"							
18	3.1	75		11	60	"			S-2.82 I-3.05	0.36 0.35	25,058 14,912	196,288 129,947	66.2
19	2.5	75		4	60	"			S-5.98 I-7.55	0.65 0.78	20,448 13,419	188,122 129,890	68.0
20	2.1	75		5	100	"			S-7.29 I-7.19	0.73 0.73	27,825 1,013	277,865 9,979	3.6
24	2.2	75		25	750	750 mg. sodium barbital			S-5.29 I-5.15	0.51 0.52	21,640 7,486	222,500 74,140	33.2
25	2.1	75	1.5	8.5	10		38		S-4.43 I-4.47	0.47 0.46	24,815 6,393	167,587 61,985	37.0
26	2.5	85	0	2.25	2.25	"	20		S-3.47 I-3.72	0.36 0.39	12,159 2,597	117,199 24,771	21.1
29	2.6	95	0.5	3.0	3.5	"	34		S-3.47 I-4.42	0.35 0.44	17,476 3,102	173,261 31,161	17.9
31	2.5	95	6.0	23.5	29.5	"	70		S-3.50 I-3.07	0.36 0.35	13,504 1,993	131,288 17,357	13.2
				45	675	"	40		S-3.69 I-3.07	0.38 0.38	18,000 18,000		

TABLE II
Sham Operation

Rabbit No.	Weight kg.	Fluid intake cc.	Urine volume			P.S.P. per cent	Anesthesia	Average blood pressure mm. Hg	Blood pressure at injection mm. Hg	Weight cortex		Weight sample gm.	Count on sample	Estimate entire kidney	Per cent patent glomeruli
			1st hour cc.	2nd hour cc.	Total cc.					gm.	gm.				
12	2.4	75			32	70	Ether			S-4.16 I-4.57		0.41 0.56	15,416 7,040	156,100 57,300	36.7
13	2.3	75			39	100	"			S-4.71 I-4.57		0.54 0.48	20,288 4,107	177,000 39,150	22.2
28	3.4	126.5	0.75	0.50	1.25	7	875 mg. sodium barbital	64	36	S-4.50 I-3.18		0.47 0.32	16,643 7,963	159,345 78,495	49.2
37	2.2	85			8.0	70	565 "	58	38	S-4.01 I-4.34		0.42 0.48	26,770 22,672	255,589 204,991	80.2
38	2.2	85	1.5	3.0	4.5	50	565 "	70	30	S-3.30 I-2.80		0.34 0.29	22,564 13,559	219,004 130,914	59.7
39	2.2	85	7.0	10.0	17.0	85	565 "	62	36	S-3.28 I-2.60		0.33 0.28	25,758 20,352	256,017 188,461	73.6
40	2.4	95	2.8	26.0	28.8	60	675 "	66	19	S-3.65 I-3.49		0.37 0.36	18,514 20,143	182,635 193,172	105.7

TABLE III

Control

Rabbit No.	Weight kg.	Fluid intake cc.	Urine volume			P.S.P. per cent	Anesthesia	Average blood pressure		Blood pressure at injection mm. Hg	Weight cortex		Weight sample gm.	Count on sample	Estimate entire kidney	Per cent patent glomeruli
			1st hour cc.	2nd hour cc.	Total cc.			mm. Hg	mm. Hg		gm.	gm.				
11	2.2	75			58	50	—				S-4.70 I-4.90		0.50 0.55	17,922 11,158	168,467 99,407	59.0
14	1.5	75			33	60	—				S-3.93 I-4.13		0.44 0.42	21,123 16,476	188,800 165,100	87.3
22	3.2	100	120—75, 18 hrs. before	35	155	80	—		70	54	S-4.99 I-5.15		0.54 0.58	19,270 7,193	178,068 63,865	36.9
23	2.2	75	20	10	30	60	—		72	84	S-4.42 I-4.25		0.47 0.48	17,262 11,760	162,338 103,700	63.8
30	2.5	95	7	10.5	17.5	80	625 mg. sodium Barbitol		50	46	S-3.73 I-3.92		0.37 0.44	25,889 7,537	260,880 67,184	25.7
32	2.4	90			7.8	60	625 " "		63	48	S-3.38 I-3.12		0.36 0.38	26,988 5,157	253,277 42,341	16.7
33	3.4	127		12	60	60	875 " "		82	66	S-4.27 I-2.98		0.42 0.29	17,757 3,280	180,100 33,704	18.7
35	2.7	100	0	0.3	0.3	0	750 " "		20	6	S-4.33 I-3.25		0.44 0.34	16,819 7,368	165,514 70,070	42.3
36	2.2	85		37	80	80	560 " "		52	22	S-3.20 I-2.59		0.34 0.28	23,289 18,596	219,190 172,012	78.4

TABLE IV
Supravital Injections

Rabbit No.	Kidney	Weight cortex	Weight sample	Count on sample	Estimate entire kidney
		<i>gm.</i>	<i>gm.</i>		
7	R	3.15	0.35	22,877	205,893
	L	2.94	0.33	22,666	201,933
31	R	3.10	0.31	17,886	178,858
	L	3.38	0.33	18,177	186,410
34	R	3.73	0.37	21,983	221,610
	L	3.32	0.33	21,511	216,411

TABLE V
Distribution of the Findings

Range of open glomeruli	Unilateral nephrectomy		Sham operation		Control	
	Open	One kidney Urine 2 hours	Open	Urine 2 hours	Open	Urine 2 hours
<i>per cent</i>	<i>per cent</i>	<i>cc.</i>	<i>per cent</i>	<i>cc.</i>	<i>per cent</i>	<i>cc.</i>
0-10	3.6	4.0				
11-20	13.2	3.5			16.7	7.8
	17.9	2.25			18.7	12.0
21-30	21.1	4.25	22.2	39.0	25.7	17.5
	23.3	0				
31-40	33.2	5.0	36.7	32.0	36.9	155.0
	37.0	10.0				
41-50	47	29.5	49.2	1.25	42.3	0.3
51-60			59.7	4.5	59.0	58.0
61-70	66.2	16.0			63.8	30.0
	68.0	11.0				
71-80			73.6	17.0	78.4	37.0
Above 80			80.2	8.0	87.3	33.0
			105.7	28.8		

RESULTS

The results on the individual animals are given in Tables I, II, III, and IV with a general summary of the urine outputs and open glomeruli in Table V.

Charts similar to Table V show that the open glomeruli vary inversely as the blood pressure, indicating that vasoconstriction for maintenance of pressure is participated in by the kidney arterioles with consequent decrease of glomerular circulation.

With the exception of four animals there is a general correlation between open glomeruli and urine output. Further the output by one kidney for a 2 hour period after unilateral nephrectomy is approximately one-half of that of the control animals. Hence, renal shock was not produced in these animals and the solitary kidney performed its share of the excretory work.

With the exception of Animal 19 with 3.6 per cent of open glomeruli, there is no essential difference in the number of open glomeruli in the normal, sham operation, and experimental groups. The normal average of 47.6 per cent is low when compared to the figures of Hayman and Starr² and Moore and Lukianoff,³ but differs by only 14.6 per cent from the experimental group. The result of 61 per cent for the sham operation animals is well within the range of normal.

The blood pressures are low but essentially the same in all three groups so that this factor would not serve to invalidate the results. The results on the control animals show that an average pressure of above 40 to 50 mm. Hg is sufficient for normal urinary output in the rabbit. Animal 17, with no output for 2 hours after unilateral nephrectomy, was not followed with blood pressure records, but it is probable that there was a low pressure. The 0.3 cc. output in control Animal 35 is accounted for on the low average pressure of 20 mm. Hg. The marked polyuria in Animal 22 is not explained. There is nothing remarkable in the other results on this animal, and the open glomeruli total only 36.9 per cent. The low output in sham No. 28 is also unexplained.

SUMMARY

1. Renal shock with oliguria or anuria after unilateral kidney operation has not been produced in seventeen rabbits.

2. For a period of 2 hours after unilateral nephrectomy the one kidney carries on a proportionate part of the work of the previous two kidneys, and there is no evidence of functional compensation.

3. There is a general correlation between open glomeruli and urine output.

We wish to thank Doctor H. T. Karsner for aid in the preparation of this report.

THE CHOLESTEROL FUNCTION OF THE GALL BLADDER

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(Received for publication, July 27, 1931)

Although cholesterol has achieved a widespread interest in relation to the general metabolism of the body it has an especial importance in surgery because of its striking tendency to localize and cause disease in the gall bladder. Gall stones nearly always contain cholesterol and many of them are composed of this substance alone. Even in the absence of stones it may be stored in large amounts under the mucous membrane, a not uncommon condition now called the "strawberry gall bladder," or more strictly "cholesterosis" of the gall bladder. Our attention has therefore been directed for many years to the behavior of this substance in the bile, especially as regards the activities of the gall bladder wall. Does the biliary epithelium excrete cholesterol in the bile, or does it absorb it? Although this question has been variously answered by writers on the subject, the problem still remains, it seems to us, an unsolved one. Evidence will be presented in this paper which, it is believed, helps to point to the true solution.

The name of Naunyn is associated with the early reports on cholesterol in relation to the gall bladder, although Virchow first suggested that the epithelium of the viscus had something to do with this fat-like alcohol. Naunyn, in 1892, first expressed the view that cholesterol in the bile is a product of the gall bladder and bile duct epithelium through desquamation and degeneration of their cholesterol-containing cells. This hypothesis was based on the study of much clinical material, on the accessory observation that inflammatory discharges from mucous membrane elsewhere (sputum) contained as much or more cholesterol than bile, and on the experiments of Jankau who found that cholesterol of the bile was independent of cholesterol intake.

Direct measurements of the cholesterol content of the bile from the gall bladder as compared with that from the liver might be expected to throw light on our problem. And many observations have been made, not only on autopsy material

of two dogs following ligation of the common duct, a condition favoring resorption whereas he saw it abundantly in two animals in which the ligature had cut through and bile was leaking out, a condition favoring excretion.

Further support for the hypothesis of excretion has been presented in preliminary studies from this laboratory (14-16) and will be described in detail in another paper (17). The experiments with cystic duct occlusion have already been mentioned. Another series of experiments in which comparisons were made between two specimens of bile collected from the same dog showed that the one subjected to gall bladder influence contained much more cholesterol than the other coming directly from the liver, even when the element of concentration of the bile was taken into account. In still other observations cholesterol was always found in the colorless secretion from the common ducts of dogs, and in the colorless hydrops fluid of the human gall bladder. Fowweather and Collinson also found cholesterol in the gall bladder contents of nine cases of hydrops as well as in the wall of the viscus, often in considerable amounts. Since these fluids are obviously the product of the biliary duct epithelium, the presence of the substance was an indication of its secretion. The present observations add further evidence in support of this view.

In this paper we shall present comparative analyses of gall bladder and hepatic bile from the same case, analyses from which, we believe, one may draw relatively accurate inferences.

Methods

Since the gall bladder has the power of concentrating bile it is necessary to measure this effect in some way. This was done by determinations of the bilirubin content, since this substance is excreted by the liver, and is indifferent to the activities of the bile duct epithelium at least under normal conditions or during short periods (38, 39). That is to say, if a particular specimen of gall bladder bile contained 3.0 mg. per cc. and the hepatic bile from the same case but 1.0 mg. per cc. it is obvious that the gall bladder had concentrated the bile three times and that if its cholesterol content was less than three times that of hepatic bile some had been absorbed; if more than three times some had been excreted.

In the analyses of human bile (Table I) the specimen from the gall bladder was obtained at operation; the hepatic bile was collected in the first three cases subsequently through the catheter placed in the common duct. Putrefaction was minimized by removing the accumulated secretion frequently and storing it in the ice box. In three cases sufficient bile was obtained for analysis by puncture of the common duct at operation, and in one case at autopsy.

In the analyses of dog bile (Table II) the gall bladder was removed and its contents were emptied immediately. The drainage of hepatic bile through intubation of the common duct was effected through the closed, 24 hour, aseptic method first described by Rous and McMaster (38). All bile specimens were centrifuged or filtered before analysis.

The chemical method for determination of bilirubin was that of Hooper and Whipple as modified by Rous and McMaster (39). Ordinarily 1 cc. of bile was added to 50 cc. of 95 per cent alcohol (containing 8 cc. concentrated HNO_3 and 40 cc. HCl per liter) and when the color turned green, was read in a colorimeter against an inorganic standard (10 cc. of 10 per cent CuSO_4 + 0.075 cc. of 1 per cent $\text{K}_2\text{Cr}_2\text{O}_7$) which in its color strength was equivalent to a solution containing 0.020 mg. of bilirubin per cc.

Cholesterol was determined by a modification of the Autenrieth-Funk method. Saponification was first effected by heating 5 cc. of bile with 20 cc. of 3 per cent KOH (in 95 per cent alcohol). Extraction was carried out in a separatory funnel with petroleum ether which was then evaporated. The residue was extracted with chloroform into 10 cc. volumetric flasks, 2 cc. acetic anhydride and 0.1 cc. concentrated H_2SO_4 added, made up to the mark, and the green color matched against a known cholesterol standard carried through the same color reaction. The details of this method are described at some length in a forthcoming paper (18).

FINDINGS

The results of our analyses are tabulated in the accompanying tables. Table I includes the human cases. These were patients operated on for cholecystitis with or without stones. It will be seen that the gall bladder bile contained much more cholesterol than the hepatic bile, the percentage differences being tabulated in the fourth column. That this increase could not be explained by concentration alone is shown by the bilirubin figures in the next columns, for the increase in cholesterol was much greater than that of bilirubin in all but one specimen. In three specimens, in fact, the hepatic bile was more concentrated than its corresponding gall bladder specimen. This is not surprising for the latter had come from a diseased organ which had lost most of its concentrating power by disease, and may even have diluted the bile by an abnormal secretion of mucus.

In four cases the comparisons were made between specimens obtained simultaneously, (three at the time of operation and one at autopsy) for it was possible in them to obtain enough hepatic bile for analysis by puncture of the common or hepatic duct. The findings are of special importance for this reason and in each case showed, as in the others, a greater cholesterol content in the gall bladder bile than can be accounted for by inspissation alone. It may be objected that hepatic bile obtained by puncture of the common duct may have been mixed with gall bladder bile passing into it through the cystic duct

CHOLESTEROL FUNCTION OF GALL BLADDER

TABLE I

Showing the Actually and Relatively Greater Cholesterol Content of Human Gall Bladder over Hepatic Bile in the Same Patient

Case	Cholesterol			Concentration of bile (Bilirubin)			Description
	Hepatic bile	G.B. bile	Difference over hepatic bile	Hepatic bile	G.B. bile	Difference over hepatic bile	
	mg. per cc.	mg. per cc.	per cent	mg. per cc.	mg. per cc.	per cent	
J. S.	— 0.11	0.50 —	+350	— 0.50	0.87 —	+74	Gall bladder removed at operation and contained many stones. Hepatic bile from drainage of common duct (4th day)
M. J.	— 0.30 0.28 0.45	3.7 — — —	— +1130 +1140 +720	— 3.2 8.1 0.67	2.4 — — —	— —25 —59 +226	Gall bladder removed at operation and contained many pigmented stones. Hepatic bile from drainage of common duct (1st, 2nd, and 6th day)
C. S. H.	— 0.63 0.21	1.2 — —	— +90 +470	— 0.25 0.45	0.65 — —	+160 +44	Gall bladder removed at operation. Many cholesterol stones therein. Hepatic bile from drainage of common duct (1st and 2nd day)
R. L.	2.8	4.8	+71	1.25	1.38	+11	Gall bladder bile from gall bladder removed at operation. Hepatic bile from puncture of common duct at operation. Both biles contained cholesterol sediment; both filtered before analysis
L. L. E.	0.74	0.19	0.15	0.00			Gall bladder removed at operation and contained colorless secretion (hydrops) and cholesterol stones. Hepatic bile obtained from puncture of common duct
M. R.	0.91	1.85	+105	0.31	0.40	+33	Gall bladder removed at operation. Hepatic bile aspirated from common duct

TABLE I—*Concluded*

Case	Cholesterol			Concentration of bile (Bilirubin)			Description
	Hepatic bile	G.B. bile	Difference over hepatic bile	Hepatic bile	G.B. bile	Difference over hepatic bile	
	mg. per cc.	mg. per cc.	per cent	mg. per cc.	mg. per cc.	per cent	
	0.94	1.85	+97	1.1	0.40	-64	Same case (M.R.) Hepatic bile from drainage of common duct (8th day)
J. B.	1.72	3.35	+94	3.7	4.4	+19	Specimens obtained at autopsy. Death due to heart disease. Biliary tract normal

TABLE II

Showing the Actually and Relatively Greater Cholesterol Content of Gall Bladder over Hepatic Bile in the Same Dog (Normal Secretions)

Dog	Cholesterol			Concentration of bile (Bilirubin)			Remarks
	Hepatic bile	G.B. bile	Difference over hepatic bile	Hepatic bile	G.B. bile	Difference over hepatic bile	
	mg. per cc.	mg. per cc.	per cent	mg. per cc.	mg. per cc.	per cent	
21	0.19	0.64	+230	0.11	0.08	-27	Bile from gall bladder removed at operation. Hepatic bile from aseptic 24 hour drainage of common duct (average of 5 days collection)
18	0.075	0.35	+370	0.081	0.083	+2	Same experiment. Hepatic bile average of 10 days collection
15	0.040	0.25	+511	0.084	0.149	+77	Same experiment. Hepatic bile average of 30 days collection
10	0.17	1.00	+490	1.82	0.98	-46	Same experiment. Average of 4 days collection

by pressure during the operation or by contraction of the viscus. If this really did occur it would have minimized the differences found and correction for it, if possible, would make the findings more striking as evidence that the gall bladder excreted cholesterol.

The findings in similar analyses in dogs are summarized in Table II. The specimens were all sterile. Here the same differences noted in man were also found. The gall bladder bile in each case contained much more cholesterol than the hepatic bile, even if we allow for inspissation of the bile as measured by the bilirubin content. In two of the dogs, in fact, the gall bladder bile was less concentrated than the liver bile. This was true because these dogs had not been fasted and the operation was performed during the height of digestion, a time during which gall bladder bile is always dilute. Yet, even in the absence of concentration the cholesterol content was quite high, an observation which we have made in numerous analyses of isolated specimens of normal dog gall bladder biles. The values for the hepatic bile were obtained by averaging the results of many determinations. In this way changes due to the operation, diet, etc. were somewhat leveled out. It must be admitted, however, that these biles, collected after an operation, are not normal like the gall bladder bile removed from the intact gall bladder. The differences found, however, were so striking that one cannot escape the inference that cholesterol is added to the bile by the gall bladder wall.

The values recorded in these experiments for hepatic bile in the dog are somewhat lower than those reported by Doyon and Dufourt, and considerably lower than those reported by Jankau. Both of these observers used older methods of analyses however. Enderlen, Thannhauser, and Jenke using the digitonin method find lower values for dog bile which agree fairly well with our results (0.08 to 0.2 mg. per cc.). Stern in 1928 found a similar range of values. McMaster's values however were, in general, somewhat higher than ours.

DISCUSSION

The analyses reported herein are the first ones (except those on the dog of Doyon and Dufourt) in which gall bladder and hepatic bile from the same source have been compared. Errors due to cellular or other debris were ruled out by filtering all specimens, and the concen-

trating influence of the gall bladder was accounted for by bilirubin determinations in each case. That in spite of these corrections the gall bladder bile still showed the greater content of cholesterol points to the wall of the viscus as its probable source. A possible objection to such an inference may be that the compared specimens, though from the same source, were not collected simultaneously, that one should really have a sample of the hepatic bile which had gone into and had been acted upon by the gall bladder, rather than that which was secreted afterwards. This would introduce a considerable error only if the liver bile varies greatly from day to day, a condition which does not occur normally. This objection applies least to the four human cases in which the two specimens were obtained simultaneously. In the case of the dog averages of many hepatic bile samples probably corrected for some of such errors. But the objection is a valid one and the findings have value as suggestive evidence only because they are so marked and consistent. Taken moreover with the results of the other experiments already pointed out, the inference seems justified that cholesterol is excreted by the gall bladder mucosa.

Since biliary epithelium is derived from the intestine this phenomenon is not surprising. Evidence has been accumulating that the intestinal tract and not the bile is probably the main site of origin (in addition to the food) of sterols found in the stool. Thus Sperry has shown that in dogs with bile draining to the outside and fed a cholesterol-free diet, the fecal output of this substance is maintained and even increased. Loops of bowel isolated from the rest of the intestinal tract have been shown to excrete perceptible amounts of fat (1) and while cholesterol was not tested for, it seems likely that it was present.

That infection increases the excretion of cholesterol has been suggested in previous experiments. Inflammatory exudates have long been known to contain large amounts of it (49). Herter and also Thomas have shown that inflammation of the gall bladder and bile ducts leads to a marked increase in cholesterol content of the bile they drain. And Illingworth was able to produce cholesterosis of the gall bladder by infecting that organ in rabbits while they were on a high cholesterol diet. It has been generally assumed that this increase coincidental with infection is due to the presence of degenerating

cells which contain cholesterol (49). There is some evidence to show that this is not entirely true (50). Although it would seem that inflammation can increase the output of cholesterol in the cell-free contents of the inflamed gall bladder or biliary tract its final proof must remain for further study.

The temptation to apply the present findings to the long discussed problem of the formation of gall stones cannot be easily resisted. If we correlate them with certain observations of others an attractive hypothesis is suggested. Thus Rosenthal and Licht have recently shown that bile salts are normally absorbed by the gall bladder wall and that inflammation accelerates this absorption, a mechanism directly opposite to the one we believe is true for cholesterol. The rôle of bile salts in keeping cholesterol in solution is plain from many observations. Oliver for example has studied 75 cases of human gall bladder bile and found that spontaneous cholesterol precipitation on standing occurred only in those with a low bile acid content and never in those with a normal or high content. With this in mind conditions leading to the precipitation of cholesterol could theoretically develop even on the basis of stasis alone. Thus bile by remaining in the gall bladder loses bile salts and gains cholesterol, conditions which if prolonged might lead to a relative supersaturation of the latter and then perhaps to its eventual precipitation.

The oft disputed question as to whether stasis alone may actually be responsible for the formation of gall stones cannot be answered on the basis of our present observations, suggestive though they are, inasmuch as certain clinical facts seem to argue against it. Thus it has been pointed out by Sherwood Moore that gall stones are rarely found in the type of patient who, by cholecystography, shows stasis in the gall bladder. This individual, of the thin asthenic habitus, often fails to empty his gall bladder for 36 hours following a cholecystogram despite a fat meal. Yet he is not the person who is apt to suffer from gall stones. It is the robust, hypersthenic one, whose gall bladder empties quickly, who most frequently has cholelithiasis. Again, we rarely see gall stones form in the thick tarry bile removed from the dilated gall bladder of patients with malignant obstruction of the lower end of the common duct, though here stasis is prolonged and complete.

It may be necessary therefore to invoke the aid of inflammation after all and the evidence mentioned seems to favor this possibility for the excretion of cholesterol as well as the absorption of bile salts by the gall bladder seems to be accelerated in the presence of infection. Various other factors are also concerned in the solution and precipitation of cholesterol, a discussion of which can be found in the paper of Lichtwitz. More recently the reaction of the bile has been shown to be of considerable importance (12) and it may be that only after the importance of all these factors are evaluated will the true pathogenesis of gall stones be revealed.

The development of a "strawberry" gall bladder is also easy to explain on the excretion theory and will be discussed at some length elsewhere (17). Cholesterol accumulates in the walls of these gall bladders presumably because the bile can no longer take up any more of it. Indeed the few analyses of gall bladder bile from cases of "strawberry" gall bladder (our own and those of Illingworth) show a high cholesterol content. Unfortunately no analyses of bile salts on these cases have been made. Corroborative of this theory too are the extensive analyses of Fowweather and Collinson who found a "mean" cholesterol value in the bile of diseased gall bladders of 3.5 mg. per cc. as compared with the normal of 2.3. They also found that the diseased gall bladder itself contained more cholesterol *i.e.* 1.20 mg. per cent against the normal of 0.91.

These studies as well as others elsewhere enable us to picture the gall bladder not as a simple viscus absorbing water and excreting mucus but as also excreting cholesterol, absorbing bile salts (37), calcium (13, 33), and as making the bile more acid (12). To explain fully its various pathological features we must take into account all these functions, for perversion of one or more may lead to a variety of results. From a simple concentrating reservoir it would seem that the gall bladder is coming to attain the manifold activities of a real abdominal organ.

SUMMARY

Cholesterol determinations of gall bladder and hepatic bile obtained from the same source reveal a greater concentration in the former even after the inspissating effect of the gall bladder is allowed for. This

evidence together with that from other experiments indicates that the gall bladder has the power to excrete cholesterol into its lumen. There is evidence also that infection may accelerate this excretion. An hypothesis is presented to explain the precipitation of cholesterol in the bile, and the bearing of these findings on the pathogenesis of cholesterol stones is briefly discussed.

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FACTORS INFLUENCING THE CORRELATION BETWEEN DIFFERENCES OF ELECTRIC POTENTIAL IN THE SKIN OF HUMAN SUBJECTS AND BASAL METABOLISM

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PLATE 57

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In a preliminary report on the relationship between electric differences of potential in the skin and normal basal metabolism we (4) concluded that, within the range of normal basal metabolism (+10 per cent to -10 per cent), there appears to be a definite correlation between the basal metabolic rate and the differences of electric potential in the skin of human subjects provided there is no marked impairment (either retardation or acceleration) in the circulation of the blood or in the physiologic functioning of the skin. We have continued our investigations regarding the factors which influence the correlation between the cutaneous electric differences of potential and basal metabolism.

Lund in 1927 and 1928, in his researches on the respiration of cells reported that the electric polarity of a cell bears a quantitative relationship to the respiratory changes of the cell. Since electric currents accompany cell oxidation, he believed that these currents were the result of oxidation-reduction potentials developed by the respiratory mechanism of the cells.

Aveling and McDowall, from a study of the cutaneous blood vessels of animals, concluded that the resistance of the skin may be used as an indication or measurement of its vascularity. A decrease in resistance was associated with vasoconstriction, and vasodilatation was associated with an increase in resistance. Wells supported these conclusions by his observations on the skin of human subjects.

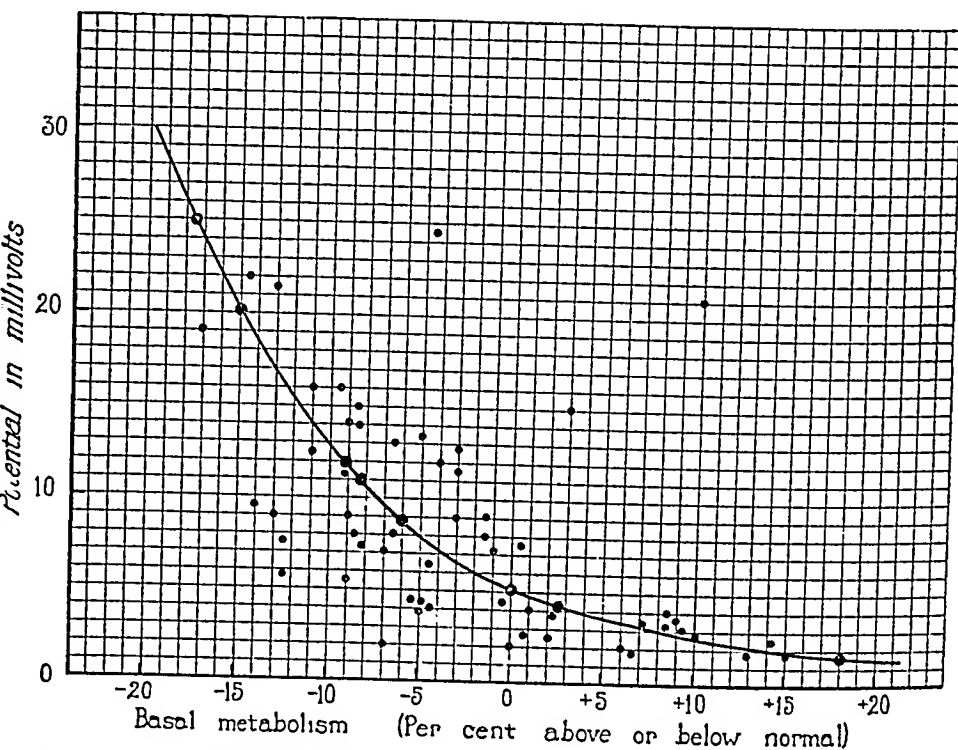
At an earlier date Waller concluded that there was a close association between nutritional changes and the values of the electric resistance of the skin, but cor-

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relation was not attempted between electric conductance and general metabolic activity.

EXPERIMENTAL PROCEDURES AND RESULTS

In Text-fig. 1 are plotted the values of the differences of electric potential as ordinates with the corresponding basal metabolisms, expressed in percentages, above or below normal, as abscissae in a group of persons presumably normal.



TEXT-FIG. 1. Correlation between the differences of electric potential and the basal metabolic rates in a group of normal persons.

The figure indicates a general tendency to grouping with some marked deviations. The curve, which was drawn from a consideration of all the data, is of the general type

$$y = b e^{ax} \quad (\text{Formula 1})$$

With the insertion of the proper constants after transformation, this equation becomes

$$x = \frac{\log y - \log 0.005}{-0.0396} \quad (\text{Formula 2})$$

where x is the basal metabolic rate and y is the difference of electric potential of the skin measured across a specified intervening area of skin. The data of Text-fig. 1 show that higher basal metabolic rates are accompanied by lower differences of potential, and *vice versa*.

Fig. 1 of the plate depicts the room and the ensemble of apparatus used in our investigations. The standard open circuit type of gasometer for the determination of basal metabolism and the apparatus containing samples of expired air collected above mercury in tubes are shown at the left of the figure. The mask is in position over the mouth and nose of the person under test. In the right portion of the illustration is shown the electric ensemble for the measurement of potential difference. The non-polarizable electrodes of Alvarez, Clark and Freeland are shown in position on the skin of the outer side of the left forearm. One electrode is placed at the articulation of the ulna and radius, while the second electrode is placed at an arbitrarily chosen but fixed distance of 12 cm. from the first electrode. Certain previous investigators believed that electric readings on the skin were influenced by sudomotor variations. We used a portion of the skin containing relatively few sweat glands in order to minimize any such effect. All measurements were made on persons who complied with standard requirements for the basal metabolic rate. Readings on the difference of electric potential were taken during the period of rest immediately preceding the test for basal metabolism. The potential differences were measured with the Leeds and Northrup potentiometer shown in the plate (at the right in Fig. 2). This instrument was used in connection with the galvanometer (Leeds and Northrup, Type R, 2,500, a) and in the plate is shown mounted on the wall in the upper right corner of Fig. 1. A portion of the electric measurements of potential were made with a combination of a portable galvanometer and millivoltmeter (left side of Fig. 2). The measurements obtained by the potentiometer and the millivoltmeter were invariably in agreement, but the portable millivoltmeter-galvanometer combination is not as readily adjusted.

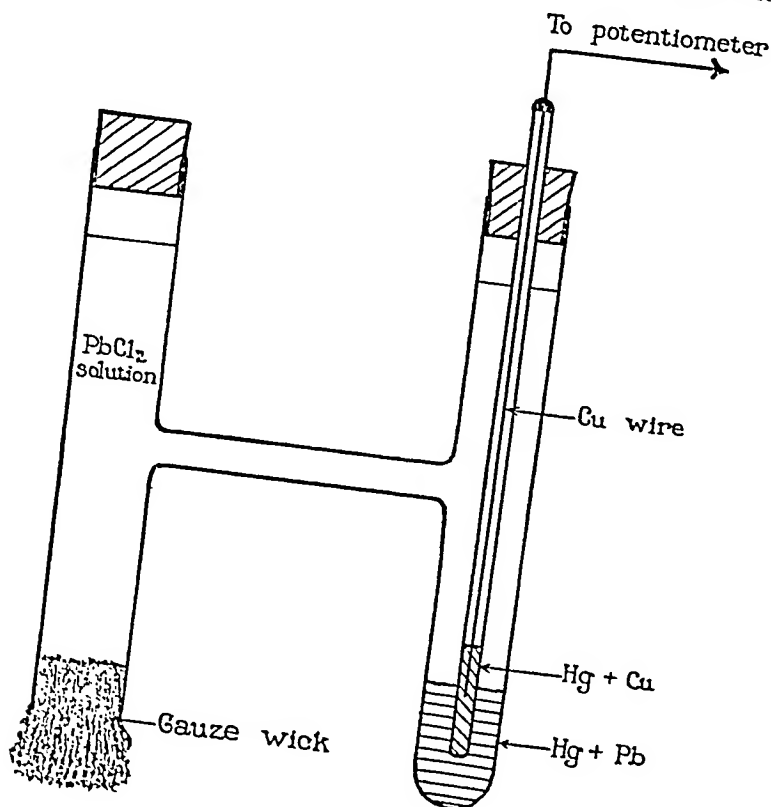
A diagrammatic sketch of the non-polarizable electrode, with indications of the materials used in its construction, is shown in Text-fig. 2. The electrode is filled with a solution of lead chloride. Electric connection with the potentiometer is made through the two mercury amalgams ($\text{Cu} + \text{Hg}$ and $\text{Pb} + \text{Hg}$). Contact with the skin is made through the gauze wick shown at the left of the figure.

After the electrodes had been in position for a few minutes (8 to 10) on the arm of the person under test, the difference of potential generally reached a fairly steady or constant value; if it did not, an average or a modal value was used. The true difference of electric potential then was obtained by subtracting the potential of the electrodes *per se* from the constant value of the potential difference in position on the forearm. Text-fig. 3 shows typical sets of data. At times there is a rise of potential difference to the steady value, as shown in Curves 1 and 2, whereas at other times, as shown in Curve 4, there is a decrease in the value of the potential difference before the constant state is reached. Curve 3 is an example taken from

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a small group of data in which the potential difference did not reach or maintain a definite constant value.

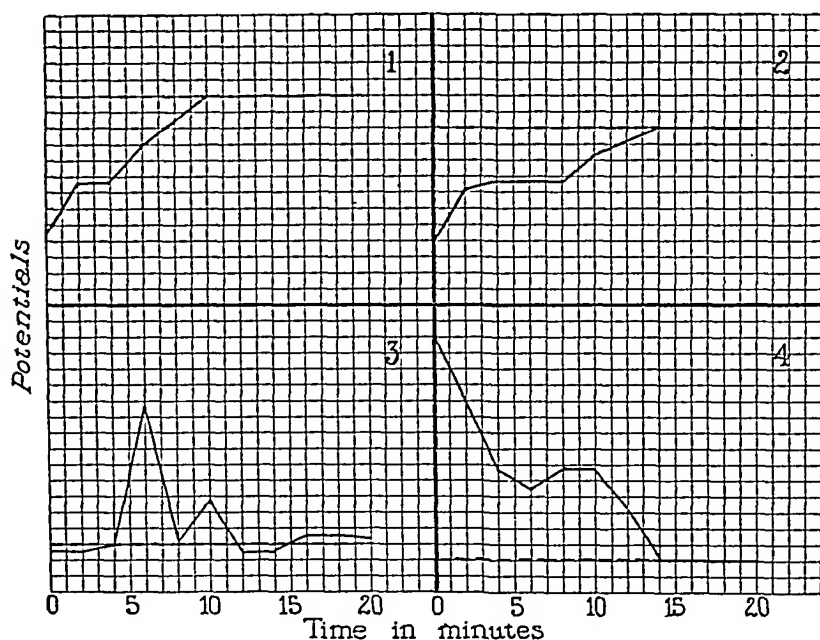
Most of the data on the basal metabolic rates were determined with an open circuit type of gasometer and analyses of the expired air by the method of Haldane. The closed circuit types of apparatus for measuring oxygen consumption (Benedict-Roth and Sanborn) also were used in these investigations. A subsequent report will be made regarding the discrepancies between the open and closed types



TEXT-FIG. 2. Sketch showing the construction of a non-polarizable electrode of instrument and the possible reasons for these discrepancies with suggestions for their correction.

Text-fig. 4 (1 to 6) shows redrawn tracings taken from tests made on a closed circuit type of apparatus: each portion is obtained during 2 minutes of the period of test. Graphs 1 and 3 exhibit exceptionally regular breathing, Graph 2 is typical of irregular or erratic breathing, Graphs 4 (hypothyroidism) and 6 evidence a slow rate of respiration, and Graph 5 is the record of the breathing of a person with hyperthyroidism.

In a previous communication we reported that the data, obtained by the methods described, were divisible into three groups: (1) rates showing a correlation between differences of electric potential in the skin and basal metabolic rates such as are exhibited by normal persons, (2) rates above the normals, and (3) rates below the normals. With few exceptions the basal metabolic rates calculated by the use of Formula 2 did not agree with the experimentally determined values of the



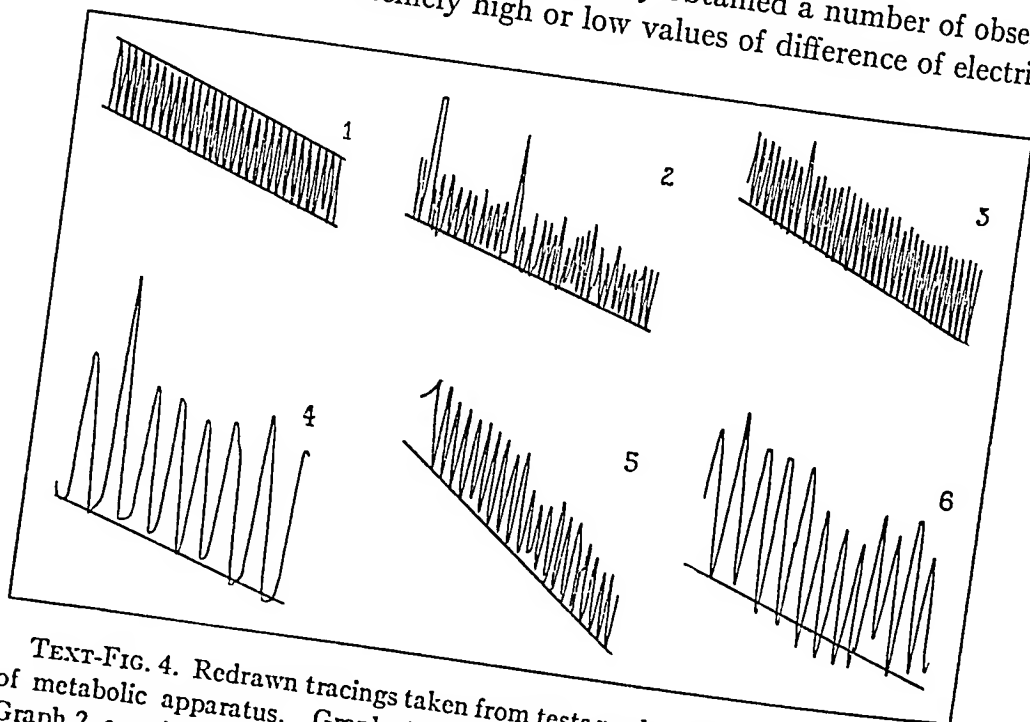
TEXT-FIG. 3. Characteristic types of curves showing the relationship between time (in minutes) and the potential (in millivolts) before the steady state is reached.

metabolic rates which fell either above +13 per cent or below -10 per cent. The agreement of rates, as determined by calculation from Formula 2 and by gasometric test, within the normal range of +10 per cent and -10 per cent, was within ± 4 points. In fact, we have used Formula 2 in attempts to predict the basal metabolism, believing that normal, healthy persons with normal circulation of blood and normal physiologic functioning of the skin manifest a definite corre-

lation between basal metabolism and differences of electric potential, and that appreciable departure from correlation is indicative of abnormality of the circulation of the blood or of the functions of the skin.

Analysis of Clinical Observations Indicative of Factors Affecting the Correlation between Electric Potentials and Basal Metabolic Rates

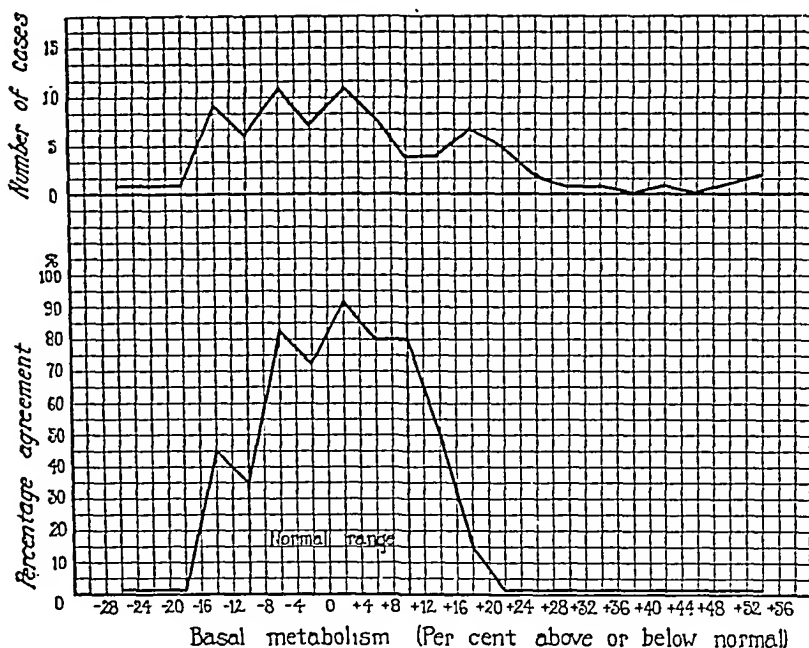
We have added to the data previously obtained a number of observations in which extremely high or low values of difference of electric



TEXT-FIG. 4. Redrawn tracings taken from tests made with a closed circuit type of metabolic apparatus. Graph 1 represents exceptionally regular breathing; Graph 2, erratic breathing; Graph 3, regular breathing; Graph 4, hypothyroidism with slow breathing; Graph 5, hyperthyroidism, and Graph 6, slow breathing.

potential or basal metabolic rates were exhibited. In almost all of these observations the metabolic rates, obtained by calculation from Formula 2 and by direct determination, have not agreed within the range of ± 4 points. The lower curve of Text-fig. 5 shows the percentage of agreement between the calculated and experimentally determined values of the basal metabolism for the number of cases in each group as shown in the upper curve of the figure. The data include

measurements on eleven normal persons and on 72 persons who had presented themselves for clinical examinations. From a consideration of the distribution of values and of the fact that we were measuring differences of electric potential and basal metabolic rates of the group of 72 persons, we were led to a survey of the records. In the basal metabolic rates of this group as determined by test, eight were



TEXT-FIG. 5. The lower curve shows the percentage of agreement between the basal metabolic rates determined by gasometric methods and from calculations on data obtained on the differences of electric potential for the number of cases in each group shown in the upper curve. (Data concerning 83 persons; 11 normal and 72 clinical subjects.)

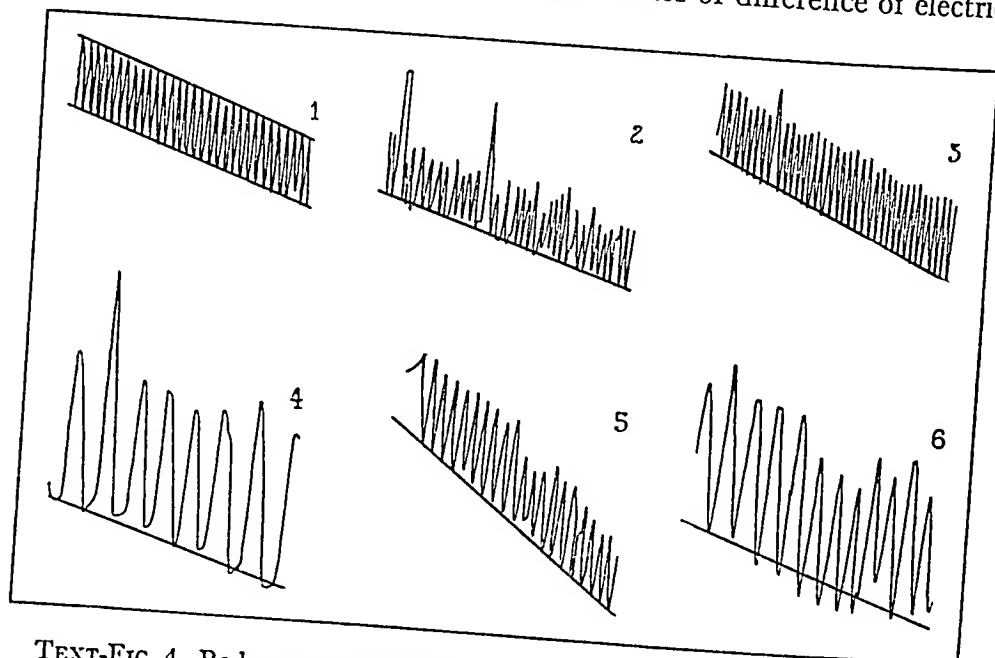
below -10 per cent, forty were within the normal range and twenty-four were above $+10$ per cent.

In cases in which the metabolic rates fell below -10 per cent there was one case only in which the rates, determined by the two methods, agreed within ± 4 points. In the clinical data regarding the other seven cases in this group there were recorded, in addition to the usual

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Analysis of Clinical Observations Indicative of Factors Affecting the Correlation between Electric Potentials and Basal Metabolic Rates

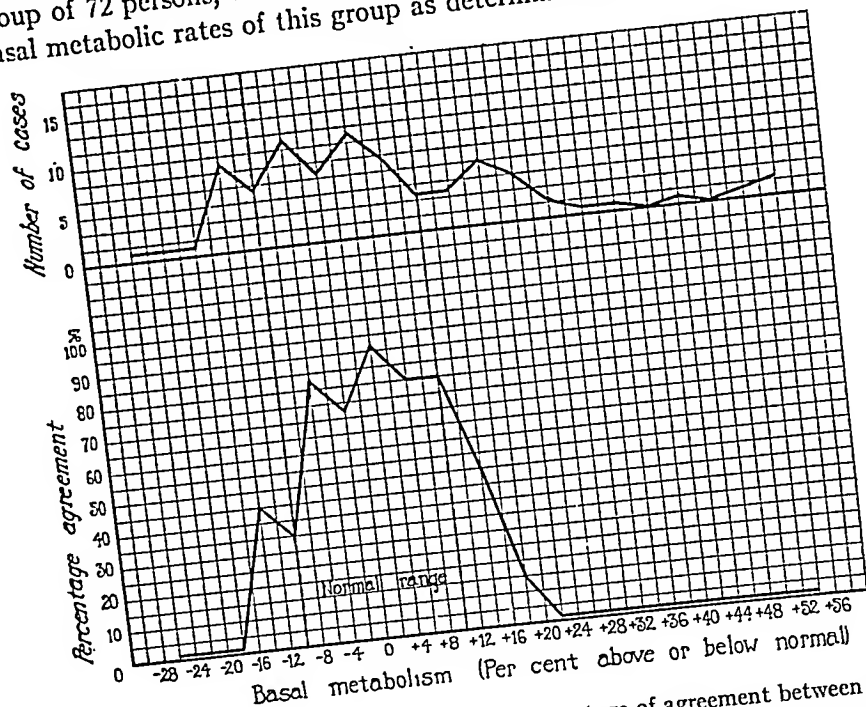
We have added to the data previously obtained a number of observations in which extremely high or low values of difference of electric



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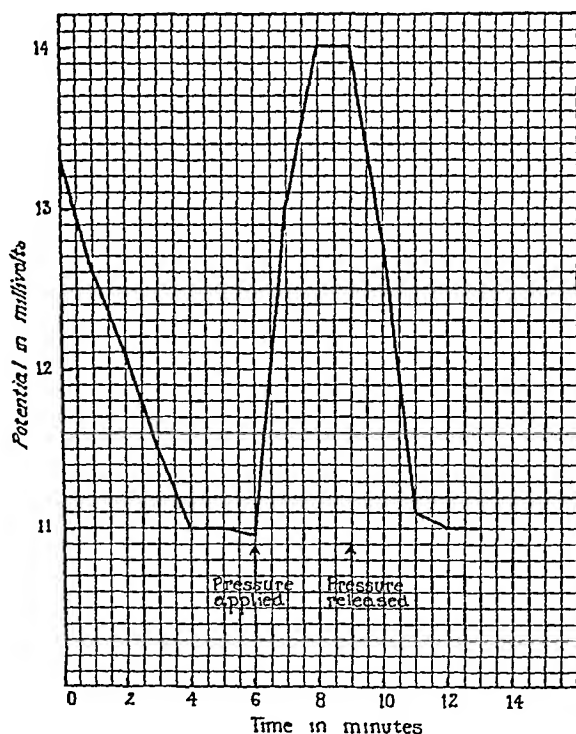
symptoms accompanying lowered metabolism (such as dry, cold skin, and so forth), migraine with edema, one case each of angina pectoris and of anemia and one case in which the patient stated that the hands and feet go to sleep frequently and easily.

There were twenty-one of the twenty-four basal metabolic rates above +10 per cent which did not agree within ± 4 points. In these twenty-one cases there were reported symptoms of hyperthyroidism, such as marked nervousness, palpitation, tachycardia or edema. A diagnosis of subacute lymphatic leukemia was made in the case of one person whose basal metabolic rate was high but who had no symptoms of hyperthyroidism. Besides the symptoms of hyperthyroidism there were reported in this group five cases of hypertension. Chronic nervous exhaustion, endocarditis, pericarditis, myocardial insufficiency, aortic sclerosis, secondary anemia and migraine also were noted. Five of the persons in this group had been placed on treatment with iodine for some time previous to the metabolic tests, hence the rates as determined may be considered as artifacts.

Within the normal range there were ten exceptions to the agreement in metabolic rates by the two methods. In one of these exceptions the metabolic rate was taken shortly after thyroidectomy; in another the rate was associated in all probability with a condition of dyspnea. Two persons reported amenorrhea and frequent fainting spells. Two patients had been on treatment with thyroxine for some time, one of these had been given a diagnosis of myxedema with hypertension and cardiac hypertrophy, and the other had taken considerable amounts of calomel during the 2 years immediately preceding, in which she had suffered from cholecystitis and anorexia nervosa. The history of one of the patients in this group carried a diagnosis of colloid goiter and mitral stenosis. In another instance, aortic sclerosis and diabetes mellitus were reported. One man, with symptoms of hyperthyroidism and pericarditis, had a low metabolic rate due to the administration of iodine; previously he had had two attacks of lobar pneumonia with circulatory disturbances and slow recovery to normality in this particular. One case of adenomatous goiter and cholecystitis also was found in this group, but there was nothing of significance by way of an explanation for the disagreement between

the metabolic rate as determined from the electric potentials in the skin and the directly determined value.

As a further check on our investigations, the data regarding persons whose metabolic rates as determined by the two methods were in agreement within ± 4 points were examined. Four persons had a rate



TEXT-FIG. 6. Curve showing the effects of the application and release of pressure on the values of the differences of electric potentials (in millivolts) across a given area of skin.

outside the normal range. One person had a rate of -12 per cent and was given a diagnosis of adenomatous goiter and cholecystitis. Three persons had metabolic rates above normal, between $+10$ and $+18$ per cent, which for purposes of this investigation, may be considered as of negative significance. In the large group within the normal range, the only cases of significance were those of mild hyper-

tension, one case of moderate aortic sclerosis and several cases of adenomatous goiter without hyperthyroidism.

In general, therefore, these investigations show that there are abnormalities in the circulatory system of the blood or in the functions of the skin of persons for whom the metabolic rates determined by the two methods (electric potentials in the skin and gasometric) do not agree. Since abnormality of the circulation of the blood and of the functions of the skin accompanies high and low metabolic rates in which there is marked disagreement, it is probable that such dysfunctions may serve as an explanation of the few instances in which the observed metabolic rates within the normal range did not correspond to the values predicted from the measurements on the differences of electric potentials.

Hyperthyroidism, as manifested by high basal metabolic rates, is accompanied by acceleration of the circulation of the blood, whereas retardation in the circulation is accompanied by hypothyroidism and low metabolic rates. We have made experimental tests which definitely show the marked changes in electric potentials caused by artificially produced changes in the normal circulation of the blood. Text-fig. 6 shows that there was a rapid increase in the value of the difference of electric potential across two chosen areas of the skin of the forearm when the circulation was temporarily cut off with pressure produced in the sphygmomanometric cuff. The difference of electric potential returned to its former normal value quickly after the release of the pressure.

Other deductions presumably may be drawn from the data thus far obtained. An inverse correlation apparently exists between cutaneous temperature and differences of electric potential. This deduction suggests the possibility of other investigations which may be of clinical significance. Furthermore, there is a correlation between the difference of electric potentials and the metabolic rates which seems to obtain under other than basal conditions. Eating breakfast and exercise do not appear to change the relationship. Day by day variations, temporary emotional changes and the partaking of food seem to affect the predicted rates less than they affect the usual (gasometric) observed rates. One example from several that might be cited clearly shows the part that emotion may play. It is that of a woman

whose first basal metabolic rate was recorded as -20 per cent with a subsequent rate of -7 per cent, whereas the rate predicted from the measurements on electric potentials, Formula 2, made just prior to the first test gave a value of -8 per cent.

Recently we have carried on a series of investigations in which we obtained data on metabolic rates under other conditions than basal, differences of electric potentials at various periods of the day and under both constant or varied environmental conditions, blood pressure, pulse rate, respiration and cutaneous temperature. The results and conclusions from these data concerning normal persons and persons presenting abnormal conditions of skin and of the vasomotor and circulatory systems will be presented in another study.

SUMMARY

High metabolic rates are associated normally with small differences of electric potential, whereas low metabolic rates are associated with large differences of electric potential as measured on the extremities of the body.

Within the normal range of metabolism there appears to be a definite correlation between the metabolic rates and the difference of electric potential over a specified area of the skin, provided the person under test has no abnormalities of the circulatory system or of the functions of the skin.

If there are no dysfunctions of the circulation or of the skin, the metabolic rate may be calculated, within ± 4 points, from the expression

$$x = \frac{\log y - \log 0.005}{-0.0396}$$

where x is the metabolic rate and y is the difference of electric potential across the specified areas of skin (electrodes 12 cm. apart).

In general, there are abnormalities of the circulation of the blood or of the functions of the skin of persons for whom the metabolic rates determined by the two methods (difference of electric potentials and gasometric procedures) do not agree with ± 4 points.

Manifest retardation or return to normality in the rate of circulation of the blood, such as may be produced by the sphygmomanometric cuff under varying pressures, produces marked changes in the difference of electric potentials obtained across a specified intervening area

of skin. Retardation of flow of blood produces increased difference of electric potential.

Preliminary investigations indicate that there is an inverse correlation between cutaneous temperatures and differences of electric potential.

Day by day variations, emotive effects and the partaking of food have less effect, in general, on the electric potentials across a specified area of skin than they have on the metabolic rates.

These experimental results indicate that there may be a more direct correlation between electric potentials and the circulation of the blood *per se* than between electric potentials and the metabolism of the body *per se*. When normality of circulation of the blood and of the functions of the skin exists in the areas under test for differences of electric potential, there is apparently a correlation between metabolic rates and electric potentials.

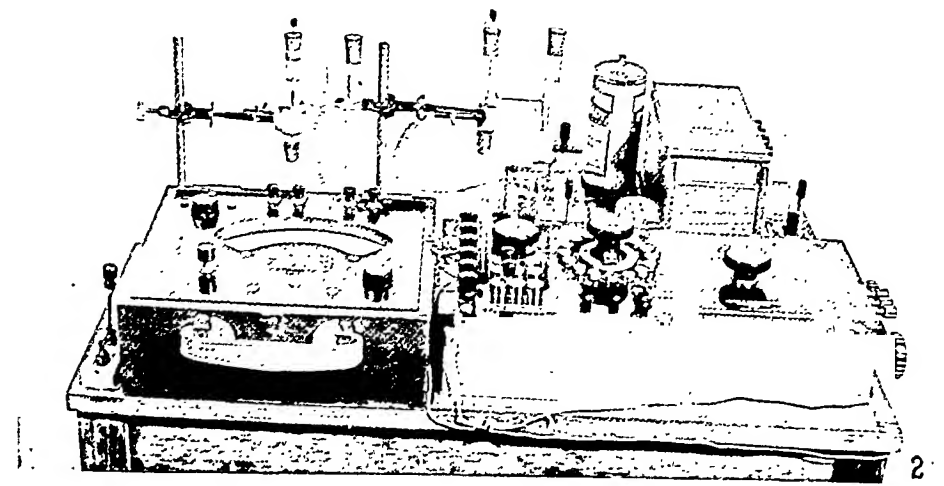
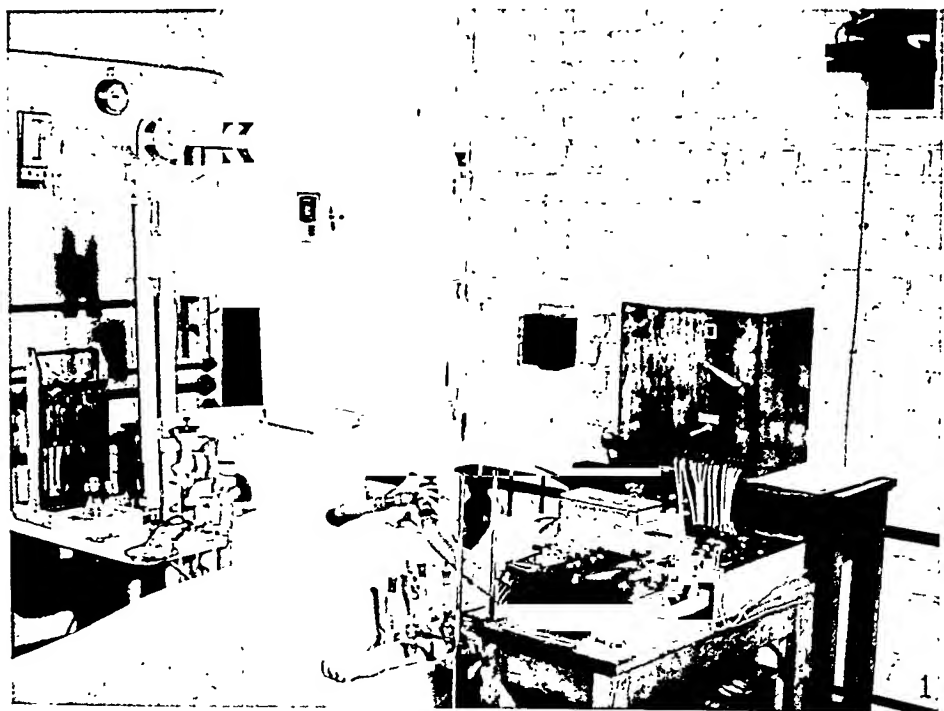
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EXPLANATION OF PLATE 57

FIG. 1. Ensemble of apparatus for the determination of basal metabolic rates by gasometric methods (left side) and by the differences of electric potential (right side).

FIG. 2. Millivoltmeter and non-polarizable electrodes (left side) and the potentiometer (right side) used in the measurements of electric potentials.



(Purdy and Sheard: Electric potential and basal metabolism)

A BACTERIOLOGICAL STUDY OF INFECTIOUS LARYNGO-TRACHEITIS OF CHICKENS¹

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Since its first recognition in 1924 as a distinct disease of chickens, infectious laryngotracheitis, more commonly termed infectious bronchitis, has assumed a position of major economic importance to the poultry industry of the United States and Canada. It is not known to have occurred elsewhere in the world.

From 1924 to 1926 studies of the disease were reported by Gwatkin (1) in Canada, May and Tittsler (2) in Rhode Island, Eriksen (3) in Missouri, Hinshaw (4) in Kansas, and Beach (5) in California. By these studies the infectious and distinct nature of the disease was established. The causative agent was found to be contained in the exudate which accumulates in the larynx and trachea of infected chickens but its presence in other organs was not demonstrated. Transmission to susceptible chickens was readily accomplished by intralaryngeal or intratracheal inoculation with exudate from the larynx and trachea of infected fowls. Attempts to produce the disease by subcutaneous, intravenous, intramuscular, or intraperitoneal injection of tracheal exudate, however, yielded either entirely negative or questionable results. Efforts by ordinary bacteriological procedures to isolate from diseased chickens any species of bacteria with which the disease could be reproduced were uniformly unsuccessful. Similar results from studies of the disease were reported by Kernohan (6) in 1930. He also reported inability to produce disease with material after passage through Seitz, Berkefeld W, Chamberland F, or Mandler filters.

For the studies reported in this paper chickens were infected by means of intratracheal injections with one or another of four strains of the causative agent of the disease. The original infective material

¹ The studies presented in this paper were briefly reported in *Science*, 1930, 72, 633.

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consisted of exudate from the trachea of diseased fowls from two farms in New Jersey and from two farms in California. It was not used for the experiments until it had been passed at least twice through chickens in which it produced characteristic and uncomplicated symptoms and lesions. The manifestations of disease in the experimental fowls were uniform for this reason, and the interpretation of the results of the experiments was not confused by the occurrence of an intercurrent infection. The sole source of chickens for the experiments was an inbred strain of Rhode Island Reds that had been neither affected with nor exposed to any infectious disease for several generations. Through the cooperation of Mr. Raymond Ring a supply of chickens from this source was continuously available.

The extremely contagious nature of the disease made it unusually difficult to avoid accidental cross-infection among the experimental animals. For this reason, the means that were successfully employed in preventing such cross-infection are worthy of brief description.

The metal cages used were sterilized by boiling. The caged chickens were kept in isolation units unconnected with each other, each having its own equipment of utensils and of overalls, rubber gloves, and rubber overshoes. These were invariably worn by anyone who entered a unit, and the persons entering were limited to the writer, and occasionally his assistant. A unit and its equipment were never used a second time without thorough cleaning and sterilization with very hot water, procedures found through several years' usage to be adequate to prevent the transfer of infection. Chickens inoculated with different types of material were never placed together. Groups of chickens inoculated with material of the same type or with graded doses of the same material were, however, kept in a single unit, each chicken in a separate cage. In such instances the data were discarded if the time of the first indications of disease made it possible that later cases resulted from cross-infection from the first cases. In the early experiments a cage containing one or more normal fowls was placed in the same unit with each lot of inoculated chickens to provide a control on the presence of virus in the unit. This precaution was discontinued when it became evident that it was unnecessary.

Bacteriological Studies

Films and cultures were made from the laryngeal and tracheal exudates and mucosa, spleens, and livers of chickens that had died of the disease or been chloroformed after definite symptoms had developed.

The films were usually stained with methylene blue for 24 hours and with Giemsa. In some instances fresh preparations were examined by dark-field illumination. The culture media included chicken- and horse-blood agar plates, chicken- and horse-blood agar slants, chocolate agar slants, chicken- and horse-blood broth, coagulated serum slants, serum agar slants, and cooked beef heart. The last mentioned medium was the only one used in the endeavor to demonstrate anaerobic bacteria. Tubed media were usually inoculated in pairs, one sealed with wax. All plate cultures were incubated for not less than 48 hours. They were examined both with the unaided eye and microscopically. No tube culture was discarded as negative until after from 10 to 20 days' incubation and after microscopic examination of stained preparations of liquid media and of the liquid at the base of the slant surface of solid media. In the majority of instances all of the types of media listed were employed. By these procedures examinations have been made of tracheal exudates of 61 chickens, of the spleens of 67 chickens, and of the livers of 48 chickens.

Results of Examinations of Tracheal Exudates.—In twenty-four instances no bacteria were observed in the stained films of the exudate. The films of tracheal exudate from thirty-seven chickens contained varying numbers of cocci and rod-shaped organisms of varying size but there was no form that could be regarded as predominant. No bacteria were seen in dark-field examinations of fresh preparations unless they were also present in stained preparations of the same material. Spirochetes, which Gibbs (7) reported to have been present in the larynx and trachea of half of forty-two chickens with infectious laryngotracheitis which he examined, were not observed.

The failure previously mentioned of a number of investigators to isolate any organism that could be regarded as the causative agent of laryngotracheitis made it evident that if the disease were due to any bacterium it was not one that could be readily cultivated. For this reason, in the examination of cultures from the tracheal exudate, attention was given only to minute or slowly developing colonies. The results obtained in cultures of the tracheal exudate of the 61 chickens were as follows:

In thirty-two instances (52.4 per cent) the bacterial growth in all cultures was so heavy and profuse that pure culture isolation was not attempted. In view of the exposed location of the exudate and of the findings in many of the films such results were to be expected.

In seventeen instances (27.8 per cent) it was possible to pick discrete,

minute colonies and therefrom to secure pure cultures. One of these proved to be a small Gram-positive coccus, one a small Gram-negative rod of the pasteurella type, and fifteen were diphtheroids. These last may be similar to the organism reported by Graham (8) as present in laryngotracheitis lesions and found by him to possess considerable pathogenicity for chickens. He stated, however, that this organism was not regarded as a primary cause of laryngotracheitis. In twelve instances (19.6 per cent) all media inoculated with tracheal exudate remained sterile. It was rather surprising to find bacteria absent from the tracheal exudate in so many cases.

Results of Examinations of Spleens and Livers.—No bacteria were observed in the stained films or in fresh preparations examined by dark-field illumination from any of the spleens and livers. The cultures from 64 spleens and 45 livers were negative. Growths of a diphtheroid type of organism were obtained in the cultures from two spleens and two livers; of a coccus from one spleen; and of a streptococcus from one liver.

Pathogenicity of the Strains Isolated.—Since, as will be shown later, it was found possible to produce laryngotracheitis by inoculation with material that was bacteria-free, the only effort to determine the pathogenicity of the strains isolated was one series of injections with a saline suspension of a mixture of ten strains. In this trial one chicken was injected with the suspension by the subcutaneous, one by the intravenous, one by the intramuscular, and one by the intratracheal method. The results were entirely negative.

Causative Agent Present in Spleen and Liver

In attempts to find the causative agent in tissues that would be more satisfactory for bacteriological examination than tracheal exudate and also to throw some light on the distribution of the virus in the body, chickens were injected by the intratracheal method with saline or broth suspensions of the spleens and livers of infected fowls. In some instances the spleen or liver of a single chicken was used and in others the organs of two or more were pooled. All tissue suspensions were carefully searched for bacteria by cultural methods as previously described.

The causative agent of laryngotracheitis was demonstrated in

eighteen of the thirty spleen emulsions and two of the six liver emulsions by the appearance of typical symptoms and lesions of laryngotracheitis in the fowls on the 3rd, 4th, or 5th day following injection. Bacteria were not found in any of the tissue emulsions which were infective. The incubation period was from 24 to 48 hours longer than that following inoculation with tracheal exudate, which was regularly 2 or 3 days; but the character of the disease was no less severe.

The adequate control of environmental conditions and the uniformity of the results made it certain that the disease which developed in the chickens following the injections of emulsions of spleen or liver resulted from the infective properties of the material injected. Furthermore, in one experiment the inoculation of fowls separately with emulsions of fresh and of desiccated portions of the same spleen gave identical results, that is to say, laryngotracheitis after an incubation period of 4 days. In four experiments, in each of which two chickens were injected with a suspension of spleen tissue, but one of each two became diseased. In two other experiments in which graded dilutions of spleen tissue were injected the causative agent was not demonstrable in dilutions higher than 1:10 and 1:50 respectively. These results show that the causative agent is much less concentrated in spleen tissue than in tracheal exudate, with which infection of fowls has regularly been accomplished with dilutions as high as 1:100,000 or 1:1,000,000. The fact that both negative and positive results were obtained with spleen tissue secured from fowls on the 2nd, 3rd, 4th, and 5th days after they had been inoculated, indicates that the time between inoculation of the fowls and removal of the spleens is not a determining factor of the presence of demonstrable amounts of virus in these organs.

The results of these experiments with spleen and liver tissue, together with the absence of lesions in any organs except the respiratory tract, indicate that the presence of the causative agent in the liver or spleen does not imply any real involvement of these organs. It seems probable that injury to the walls of the blood vessels of the larynx and trachea permits the entrance of the etiological agent into the blood stream and its distribution. Further evidence that laryngotracheitis is not accompanied by a general organic or systemic involvement is provided by the fact that no thermal reaction of significance

LARYNGOTRACHEITIS OF CHICKENS

occurred in the experimentally infected chickens. In illustration of this the temperature records of three chickens which are representative of many are given in Table I.

TABLE I
Temperature Record of Fowls after Inoculation with Laryngotracheitis

Chicken No.	Day				
	1	2	3	4	5
1740	42.2*				
1824	42.0*	42.4	42.2**	42.2***	
1845	41.8*	42.4**	42.1	41.9	42.6***
		41.8**	42.1	42.3	42.1***

* Inoculated.

** First symptoms.

*** Marked symptoms. Chloroformed.

Host Specificity of the Disease

The transmission of laryngotracheitis to birds other than chickens was attempted to determine, first, to what extent other species, particularly the wild species and domesticated but free-flying species such as pigeons, might be susceptible and therefore of importance as agencies in the spread of the infection, and second, if there were some bird host less susceptible than the chicken, by passage through which the virulence of the causative agent might be so modified that it could be utilized for the immunization of chickens.

In these experiments 5 sparrows, 1 crow, 3 doves, 1 starling, 9 pigeons, and 15 ducks were used. The wild birds were trapped on the Institute grounds; the pigeons were secured from a source that was known to be free from disease and from all contact with chickens; the ducks were hatched and reared at the Institute. The injections were entirely by the intratracheal method. The virulence of the inoculum was always demonstrated by the inoculation of susceptible control chickens.

These experiments resulted in complete failure to transmit the infection to any of the birds except the control chickens. When the initial injection into pigeons failed, an endeavor was made to enhance the virulence for these birds by serial passage. This

likewise failed. Furthermore, by the intratracheal injection into fowls of the scrapings of the tracheal mucosa of pigeons that were chloroformed on the 5th, 8th, or 10th days after injection it was determined that the causative agent had not survived in the tracheas of the pigeons. These findings are not in accord with Kernohan's (6) report of the observance of spontaneous infection with laryngotracheitis in sparrows and pigeons on infected poultry farms.

Attempts have been made to transmit laryngotracheitis to rabbits, guinea pigs, and white rats, by intratracheal, intracerebral, and intravenous injections, and to one pig by intratracheal injection of tracheal exudate taken from diseased fowls. The presence of the causal factor in the material injected was demonstrated in all instances by its ability to infect control chickens. None of the animals proved susceptible to the disease.

SUMMARY AND CONCLUSIONS

1. The causative agent of infectious laryngotracheitis of chickens was found to be present in bacteriologically sterile tracheal exudate, spleens, and livers of diseased fowls.

2. The causative agent was present regularly in the tracheal exudate, in the spleens of about 60 per cent, and in the livers of about 30 per cent of chickens with active laryngotracheitis infection.

3. Suspensions of the spleen and liver were less effective in inducing the disease than those made from the tracheal exudate. This finding, together with the absence of pathological changes in the spleens and livers would seem to indicate that they are not actively involved but that the causative agent is carried to them by way of the blood.

4. The disease could, in our experience, be produced only in chickens. Domesticated ducks and several wild and free-flying species of birds, including sparrows, crows, starlings, doves, and pigeons were found to be refractory, and so too were rabbits, guinea pigs, white rats, and one pig that was tested.

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3. Eriksen, S., *Yearbook Missouri State Poultry Board*, 1925, 1923-24, 48.

The filtration tests were made with exudate from the trachea of fowls that had been artificially infected with one of four strains of the disease (1). The exudate was weighed, thoroughly ground in a sterile mortar or by means of a modification of Hagan's (3) grinding apparatus, and suspended in infusion broth or other diluent. The suspensions were held in the refrigerator for about 2 hours and were then centrifuged at high speed for 10 to 20 minutes. The supernatant fluid, to which was added *B. prodigiosus*, was filtered by positive pressure through Seitz discs or by negative pressure through Berkefeld candles. The quantity of suspension used, 20 to 30 cc., usually filtered in from $\frac{1}{2}$ to 1 minute and always within 2 minutes. The negative pressure for filtrations through Berkefeld candles varied from 30 to 50 cm. The amount of positive pressure for filtrations through

TABLE I
Results of Filtration Experiment 20

Filtrate No.	Description of filters			Amount filtered	Time required	Maximum negative pressure	Results of injections					
	Grade and No.	Times used	Results of H ₂ O flow test				Chicken No.	Dose	Results	Incubation period	Days after inoculation died or killed	Amount and type of exudate in larynx and trachea
			cc.	cc.	sec.	cm.		cc.		days		
20-V-3	V-3	9	56	20	20	40	2500	0.4	+	3	K-6*	Caseous plug
20-N-3	N-3	4	68	20	20	45	2501	0.4	+	4	K-6	Profuse; very hemorrhagic
20-N-5	N-5	New	61	20	25	40	2502	0.4	—	—	—	
20-N-6	N-6	3	28	20	50	50	2503	0.4	—	—	—	
Unfiltered suspension—Control							2504	0.4	+	1	K-3	Profuse; very hemorrhagic

* K = Killed, the numeral giving the day after inoculation.

Seitz discs was not measured. The unfiltered material and the filtrates were tested by the injection of small quantities into the trachea of susceptible chickens. Data concerning the experimental fowls, and the precautions taken to avoid accidental or cross-infection among them, are described in the first paper (1).

The following protocol of filtration Experiment 20 is representative of the procedure that was followed.

Specimen Protocol.—Three chickens were given intratracheal injections of laryngotracheitis virus, Strain N. J. II. All exhibited marked symptoms on the 3rd day and were chloroformed. The tracheal exudate was removed with aseptic precautions and weighed 0.8 gm. It was ground in a sterile mortar with 80 cc. meat infusion broth. The suspension was held for 3 hours in the refrigerator

and centrifuged at high speed for 10 minutes. A small amount of the supernatant fluid was removed for use in the inoculation of control fowls and a 6 hour broth culture of *B. prodigiosus* was added to the remainder. After each of two blood agar slants had been inoculated with 1 loopful of this fluid, it was divided into four portions of 20 cc. one of which was passed through a Berkefeld V candle and the other three through three different Berkefeld N candles. Blood agar slants were inoculated with 1.5 cc. of each of these filtrates and a 5 cc. portion of each filtrate was transferred to a sterile tube and incubated. A description of the filters, the results of tests for active virus in the suspension before and after filtration, and other details are given in Table I.

Results of Filtrations

A summary of thirty-six filtration experiments, in which all four strains of the disease were used is given in Table II. In all but two

TABLE II
Summary of Filtration Experiments

Filters		Filtrations			No. of filters that gave positive filtrates
Grade	No. used	Virus	No. attempted	No. positive	
V	8	New Jersey I	1	1	8
		New Jersey II	13	10	
		California 4	1	1	
		California 5	5	2	
N	6	New Jersey II	8	4	2
		California 4	1	1	
		California 5	1	0	
Seitz	6 pads	New Jersey I	2	0	
		New Jersey II	4	0	

of the experiments the tracheal exudate was suspended in bouillon; in those two Tyrode's solution was used.

It will be seen that all of the Berkefeld V filters gave positive results while only two of the six N's allowed the etiological agent to pass. These two N's were by the Ward and Tang test as porous as the V's. Of the four filters that gave negative results one was as porous as the V's while the remaining three were less porous. The results indicate that the passage of the disease-producing agent is in some way dependent upon the grade of the filter.

Since all of the V filters gave positive results some consideration must be given to the six negative experiments. In two of them Tyrode's solution was used to suspend the exudate. As this same exudate suspended in bouillon and passed through other filters produced disease it was concluded that Tyrode's solution is not a suitable diluent. The negative results in the remaining four experiments may be due to adsorption of the etiological agent on the filters. That there is adsorption is shown by titrations of filtered and unfiltered suspensions. While from 0.0001 to 0.000001 cc. of the latter will produce disease it takes from 0.1 to 0.01 cc. of the former. Occasionally infection will follow the injection of 0.001 cc. of the filtrate.

The Seitz filters are so much more porous than the Berkefeld candles that the size of their interstices cannot account for their failure in six attempts to pass the infectious agent. It is probable that some physical chemical property of the filter pad or of the agent is responsible. Kernohan (4) has reported as a personal communication that the author has passed the causative agent of laryngotracheitis through Seitz filters. It is now believed that in the experiments referred to the chickens were infected by contact with other diseased birds rather than by the inocula.

Examination of Table II shows that of the thirty Berkefeld filtrates nineteen produced disease. It was typical in every respect except that the incubation period was from 1 to 2 days longer than that in control birds. Titration experiments referred to above with filtered and unfiltered suspensions show that a large amount of the infectious agent is lost during passage through the Berkefeld candles. It seems probable that the lengthened incubation period in the fowls receiving the filtrate was due to the small amount of the agent present.

The positive results in these filtration experiments are sufficient to establish the fact that the agent that causes laryngotracheitis in chickens is a filtrable virus.

Since viruses as a class differ from bacteria in that they are more readily neutralized by immune sera and more resistant to drying it was of interest to determine whether this virus was typical in these respects.

Neutralization Experiments

Immune sera were obtained from four chickens on the 10th or 12th day after recovery from the New Jersey Strain II of the disease. Two

of these chickens had been bled before inoculation and their sera as well as sera from other normal chickens were used as controls.

The first experiments were not so clear-cut as we desired and will not be reported in detail. In two instances there was definite neutralization but in most of the cases the onset of the disease was only delayed.

In these first experiments the virus was in fresh tracheal exudate and was used in concentration many times greater than that needed to infect. The results suggested that a more careful adjustment of the proportion of virus to immune serum would give a definite neutralization.

For further experiments one lot of desiccated New Jersey II and one of California C-5 virus were used. These two viruses were titrated in order to determine the amount necessary to produce disease. Mixtures of sera and virus were prepared in which the concentration of the latter was approximately ten times that necessary to infect. These mixtures were kept at room temperature for 2 hours before they were used for the inoculation of chickens. The amounts of sera and virus used and the results of the inoculations are given in Table III.

From the data given in Table III it is seen that chickens were not infected by the intratracheal injection of suspensions of from ten to 100 times the minimum infective dose of N. J. II or C-5 strains of virus when it was mixed with an equal volume (and in one instance with one-tenth its volume) of serum from fowls that had recovered from infection with Strain N. J. II virus. The relatively long incubation period in the chickens that were inoculated with mixtures of serum diluted 1:10 and virus suspensions indicated that partial or transitory neutralization of the virus had occurred. The disease so produced was unmodified except for the delay in onset. The effects of injection of mixtures of immune serum diluted 1:100 and virus suspension or of mixtures of normal serum and virus were not different from those of inoculation with virus suspension alone.

The finding that the serum of chickens that had recovered from infection with a New Jersey strain of virus was equally effective in neutralizing *in vitro* the New Jersey and the California strains of virus provides evidence of the etiological identity of the disease known as

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laryngotracheitis or infectious bronchitis in the two widely separated States.

TABLE III
Neutralization in Vitro of New Jersey Virus, Strain N. J. II, and of California Virus, Strain C-5 with Blood Serum of Chickens Immunized against New Jersey Virus, Strain N. J. II

Virus	Serum	Chicken No.	Amount of serum	Amount of virus suspension	Results	Incubation period	Remarks
N. J. II 1:500. Suspension	1718 Immune	2413	cc. 0.2	cc. 0.2	—	—	Fowl susceptible to subsequent inoculation with active virus
		2414	0.02	0.2	+	5	Chloroformed on 6th day. Moderate amount of slightly hemorrhagic exudate
		2415	0.002	0.2	+	3	Chloroformed on 6th day. Moderate amount of very hemorrhagic exudate
	1756 Immune	2416	0.2	0.2	—	—	Susceptible to subsequent inoculation with active virus
		2417	0.02	0.2	—	—	Susceptible to subsequent inoculation with active virus
		2418	0.002	0.2	+	3	Chloroformed on 6th day. Caseous exudate
	1718 Normal	2419	0.2	0.2	+	2	Died on 6th day. Very hemorrhagic caseous exudate
		2420	0.2	0.2	+	2	Died on 6th day. Caseous plug in larynx
	1756 Normal None	2421	None.	0.2	+	2	Died on 5th day. Profuse very hemorrhagic exudate
		2422	Virus controls	0.02	+	2	Died on 6th day. Caseous plug in larynx
		2423		0.002	—	—	Susceptible to subsequent inoculation with active virus

TABLE III—*Concluded*

Virus	Serum	Chicken No.	Amount of serum	Amount of virus suspension	Results	Incubation period	Remarks
			cc.	cc.		days	
C-5 1:5000. Suspension	1718 Immune	2429	0.2	0.2	—	—	Susceptible to subsequent inoculation with active virus
		2430	0.02	0.2	+	6	Developed marked symptoms. Allowed to recover
		2433	0.002	0.2	+	4	Died on 7th day. Profuse very hemorrhagic exudate
	1756 Immune	2431	0.2	0.2	—	—	Susceptible to subsequent inoculation with active virus
		2432	0.02	0.2	+	5	Died on 7th day. Caseous plug and moderately hemorrhagic mucous exudate
		2434	0.002	0.2	+	3	Died on 7th day. Caseous plug and moderately hemorrhagic mucous exudate
	1718 Normal	2435	0.2	0.2	+	3	Died on 5th day. Profuse very hemorrhagic exudate
	1756 Normal	2436	0.2	0.2	+	3	Died on 5th day. Profuse very hemorrhagic exudate
	None	2437	None.	0.2	+	3	Died on 5th day. Profuse very hemorrhagic exudate
		2438	Virus controls	0.02	+	3	Died on 5th day. Profuse very hemorrhagic exudate
		2439		0.002	+	4	Died on 6th day. Profuse very hemorrhagic exudate

Resistance to Drying

Although few tests on the resistance of the virus to drying have been made they show that it withstands conditions that kill most bacteria.

In one experiment exudate in tubes was kept in a desiccator containing calcium chloride at incubator temperature for 2, 4, 6, 8, and 10 days. The tubes were sealed with wax and stored in a refrigerator for 2 months at which time the material was tested and found to produce disease.

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In another experiment exudate kept in a desiccator over calcium chloride at refrigerator temperature for 29 days was found to be active.

Swift's (6) method that preserves bacteria as well as viruses has been used to keep on hand adequate supplies of virus and to obviate the necessity of continuous passage through fowls. When the exudate was dried rapidly and kept in the refrigerator in sealed tubes the virus remained alive for 156 days. No tests have been made on material stored for a longer period.

SUMMARY AND CONCLUSIONS

1. Experiments have shown that tracheal exudate from two strains of laryngotracheitis of chickens from New Jersey and two from California when suspended in bouillon and passed through Berkefeld V filters will produce the disease. Two of six Berkefeld N filters allowed the etiological agent to pass, whereas four did not. Attempts to produce the disease with Seitz filtrates were unsuccessful. These results demonstrate that laryngotracheitis is caused by a filtrable virus that because of its size or some other property does not pass readily through the finer filters.
2. It has been shown that the sera from fowls that have recovered from an infection with one of the New Jersey viruses will neutralize the same strain and also the one California strain tested. In order to demonstrate neutralization conclusively it was necessary to titrate samples of dried virus and in the tests to use approximately ten infecting doses.
3. The virus dried over calcium chloride for 10 days and then stored in the refrigerator for 60 days produced disease. Kept over calcium chloride for a month it was still active and when dried by Swift's method it remained alive for 5 months.

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HISTOPATHOLOGY OF INFECTIOUS LARYNGOTRACHEITIS IN CHICKENS

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PLATES 58 TO 60

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Since the first report of the occurrence of laryngotracheitis by May and Tittsler (1), this disease has been studied and described in various parts of the United States by Eriksen (2), Hinshaw (3), Beach (4), Graham (5), and Kernohan (6). They have dealt with the clinical symptoms, the gross pathological findings, and the infectious agent, which has been definitely proven by Beach (7) to be a filtrable virus. No histological study has been published. It is the purpose of this paper to report such studies, which were started in January, 1930, and continued in close collaboration with the etiological and experimental work of Beach.

Material

The material consisted of thirty cases of laryngotracheitis, of which fourteen were experimental and sixteen spontaneous field cases. Some of the fowls were killed with chloroform between 6 hours and 7 days after inoculation while others died in various stages of the disease. The field material and the virus with which fowls were inoculated experimentally were obtained from both New Jersey and California outbreaks.

Animals were inoculated intratracheally, intranasally, into the eye, or into the cleft palate. A few were allowed to contract the disease through contact with infected chickens. The disease picture was the same after all types of inoculation except into the nasal fossa, the eye, and the cleft palate. Infection by these routes led to the development of a more or less marked discharge from the nose, eyes, or cleft palate in addition to the typical laryngotracheitis.

Gross Pathology

Larynx and Trachea.—At autopsy the most striking lesions were regularly found in the upper larynx, the trachea, and less frequently the lower larynx. 6 to 42 hours after inoculation only a small amount of mucus was visible. Between 42 and 74 hours after inoculation there might be small hemorrhages in the mucous membrane or a small amount of mucus mixed with clotted blood and restricted entirely to the upper larynx; while 3 or more days after inoculation the mucus might be replaced by caseous, yellowish masses which collected in the larynx or trachea to such an extent that they entirely plugged the opening. The mucous membrane of the lower trachea was normal except for a slight swelling, reddening, and increase of mucus which covered it with a thin film. In most of our cases this process extended the entire length of the trachea and often into the main bronchi. The degree and the kind of lesions varied greatly. Frequently the mucous membrane was covered with a comparatively thin film of clotted blood, with mucofibrinous and caseous masses, or with a mixture of blood and exudate; while in other cases clotted blood or exudate was collected in a mass so large that it completely occluded the lumen of the lower portion of the trachea and of the main bronchi. Sometimes the amount of clotted blood exceeded that of mucus. Cross-sections through the larynx, trachea, and bronchi showed an enormous thickening of the mucous membrane.

Except for a more or less marked congestion and edema the lungs appeared entirely normal in most of our cases. In a very few a yellowish exudate between the chest wall in either or both lungs and foci of consolidation and necrosis of a yellowish color were present.

Nasal Passages and Sinuses.—A number of chickens which contracted the disease naturally or were inoculated in the nasal fossae, cleft palate, or eyes showed, in addition to the described lesions, an involvement of the upper respiratory tract. The turbinate bones and the paranasal sinuses were sometimes filled with seromucoid, water-clear or yellowish caseous material, which by application of a slight pressure could be forced out of the nostrils and cleft palate. This involvement of the nasal passages and the sinuses was often indistinguishable from that found in the common fowl diseases, A-avitaminosis, pox, roup, and diphtheria. The same inflammatory process might be present in the eyelids.

Alimentary Tract.—In a large percentage of cases, even in very early ones, the mouth cavity contained small thin patches or films, either round or oval, which could be removed without leaving defects in the underlying mucous membrane. These lesions, although easily distinguished from those found in diphtheria, are also found in common roup and A-avitaminosis and are therefore not characteristic of laryngotracheitis.

No significant gross lesions have been found in any part of the intestinal tract. Some cases showed a slight inflammation of the small intestine.

Spleen.—In a few cases the spleen appeared slightly enlarged.

Other Organs.—No gross lesions were encountered in other organs. In a number

of birds in which occlusion of the trachea or bronchi caused death by asphyxiation, small hemorrhages were found in the epicardium.

Histology

Larynx and Trachea.—24 to 72 hours after inoculation the predominant picture is a more or less marked edema and cellular infiltration of the tunica propria and the submucosa (Fig. 1). In some of our cases the edema of the mucous membrane causes an entire disarrangement of its normal structure (Fig. 2). The inflammatory process may even spread into the adventitia. One of the most noticeable lesions is the presence of small hemorrhages around smaller vessels of the propria mucosa and submucosa or such an extravasation of the red blood corpuscles that the mucosa and submucosa are completely separated from each other. A few red blood corpuscles also occur throughout the upper part of the edematous mucous membrane and even appear on its surface. In view of the negative histological findings in the walls of the blood vessels in these early stages, one is led to believe that the escape of cells is due to rupture of smaller vessels of the submucosa caused by violent coughing and respiratory efforts or possibly by diapedesis.

In later stages of the disease (3 to 7 days after inoculation) the lesions in the larynx and trachea are characterized by a dense cellular infiltration in both tunica propria and submucosa (Fig. 3). From the comparative study of a number of cases it becomes evident that the larynx as a rule shows more infiltration than the trachea and bronchi. While in some of the cases the normal structure of the mucous membrane and the mucous glands can still be recognized, in the majority there is such a dense infiltration that the epithelial elements become more or less dislocated and destroyed.

The greater number of the infiltrating cells are small lymphocytes, with histiocytes, and a few plasma cells. The proportion of the different kinds varies; while in some cases the lymphocytes are most numerous, the histiocytes predominate in others. Eosinophilic leucocytes, present normally in the trachea of chickens, may be increased in number. Mitotic figures indicate a slight increase of the connective tissue elements. In sections impregnated by Bielschowsky-Maresch's silver method, connective tissue fibers are found to be increased. The fibers are swollen and sometimes broken as a result of the enormous edema. The same is true of the elastic tissue fibers. The blood vessels show no distinct and regular lesions except for a more or less marked swelling of the endothelial cells and a change in the chromatin structure of their nuclei. In some cases retrogressive changes are found in the connective tissue cells of the submucosa, which may contain small fat droplets in their protoplasm and nuclei. This degenerative process does not usually reach a high degree during the first 5 days after infection. Older cases, however, frequently show actual necrotic foci in the submucosa and tunica propria with a general hyperchromasia, pycnosis, and karyorrhexis of nuclei, and fatty degeneration of cells including those of the various infiltrating elements. In these late cases and sometimes also in earlier ones, a marked proliferation of endothelial cells

with necrosis of the walls of the blood vessels is present. These lesions, when they occur, may provide an entrance for the virus into the blood stream and its conveyance to the liver and spleen in which, according to Beach (7), it is found frequently.

While in some very early cases the epithelium of the mucous membrane and of the mucous glands retains its normal structure and the individual cells are almost normal in appearance, more or less marked changes can usually be seen as early as the 6th hour after inoculation. The first change is a slight thickening of the surface epithelium caused by the general edema of the wall of the trachea, enlargement of individual cells, and occasionally multiplication of cells (mitotic figures). Some of the cells may appear practically normal, but the majority of those lining the upper surface lose their cilia and are thrown off, and show retrogressive changes, oftentimes the so called "balloon degeneration." Reticular degeneration of epithelial cells in the surface layers is a rare finding. In most of the cases showing a marked edema of the mucous membrane there is a disarrangement of the various parts of the tracheal wall, and in those with a pronounced infiltration the epithelium, including the mucous glands, is nearly destroyed (Fig. 3).

Formations simulating giant cells occur in the edematous fluid between the surface epithelium and the submucosa (Fig. 2). By the use of Mayer's mucicarmin stain they can be demonstrated to be mucous glands separated from the surface epithelium and scattered irregularly in the edematous mucous membrane. They are always found compressed by the surrounding edematous fluid and show degenerative changes of the peculiar type described above. Later the nuclei disintegrate, the degenerative products collect in the center, and there is a hyperchromasia and hyper eosinophilia. Finally the whole formation may be transformed into a necrotic area in which mucin can still be demonstrated with specific stains. Fat may be present in large amounts.

The individual variation in the findings is great, depending upon the length and severity of the process. Secondly invading bacteria may lead to a partial or diffuse necrosis of the tracheal wall and complicate the picture. Cocci and more frequently diphtheroids, probably similar to those described by Graham and his coworkers (8), are present in such cases. In general, however, such bacteria seem to play a minor rôle. Rickettsias have not been found.

Tracheal Exudate.—The composition of the exudate in the trachea differs widely in individual cases. In the early stages there is only a slight proliferation of the upper layers of epithelial cells which are thrown off either singly or in layers and collect on the surface of the mucous membranes; while in other cases there is a mixture of epithelial cells, mucus, round cells, eosinophilic leucocytes, and red blood corpuscles. Sometimes the cellular elements predominate, sometimes the mucus. There are cases in which the mucous membrane is covered with a thin layer of red blood corpuscles. In other cases what appears to be exudate is really edematous mucous membrane which has become separated from the underlying tissue and thrown off in large masses. It is a striking fact that fibrin could seldom be demon-

strated in the exudate. In a number of cases the exudate consists of degenerated cells and sometimes real necrosis is seen. In instances with marked infiltration of the mucous membrane exudate may be entirely lacking.

Lungs and Bronchi.—The changes in these organs are usually restricted to the bronchi and the tubules. The bronchitis, which is almost always present, varies in degree and is often associated with a peri- and parabronchitis. The lesions resemble those in the larynx and trachea but very seldom are as marked. The bronchial exudate is largely made up of the same elements and shows the same variations as that in the trachea. Sometimes bronchiectasis is present. In addition to these lesions, in almost all cases there occurs marked congestion indicated by an extreme filling of the capillary network of the lungs and a more or less pronounced edema (Fig. 4). Scattered around the hemorrhages in the bronchial wall and in the congested areas are groups of hemin crystals either in the tissue or included in various cellular elements.

These lesions in the lungs and bronchi seem to be more extensive in the spontaneous disease than when it is artificially produced. In a few cases a true pneumonia is present in the tissue surrounding the affected bronchi. In addition more profuse hemorrhages and larger foci of necrosis are found in the lung parenchyma, often walled off by fibroblasts. In many cases numerous foreign body giant cells are found among the fibroblasts (Fig. 5).

Nasal Cavities and Communicating Sinuses.—The lesions in the nasal passages and the communicating sinuses vary considerably. In general, they are analogous to those previously described in the trachea (Fig. 6). Lesions due to bacterial infection are just as rare as in the trachea and bronchi.

Eyelids and Eyes.—In natural and experimental cases with involvement of the eyes, the eyelids show the same inflammatory process as the trachea. No lesions are present in the other parts of the eye.

Alimentary Tract.—The small yellowish membranes frequently seen in various parts of the cavity of the mouth are necrotic areas, sometimes very superficial, sometimes destroying the entire epithelial layer of the mucous membrane. Numerous clumps of bacteria are found which are probably the cause of the lesions. They occur in a number of other diseases, and hence cannot be considered characteristic of the infection under discussion.

The esophagus, proventriculus, and intestinal tract are free from lesions except, in a few cases, for a slight enteritis which does not seem to be specific.

Spleen.—The spleen when enlarged shows a hyperplasia without any other particular or characteristic lesions.

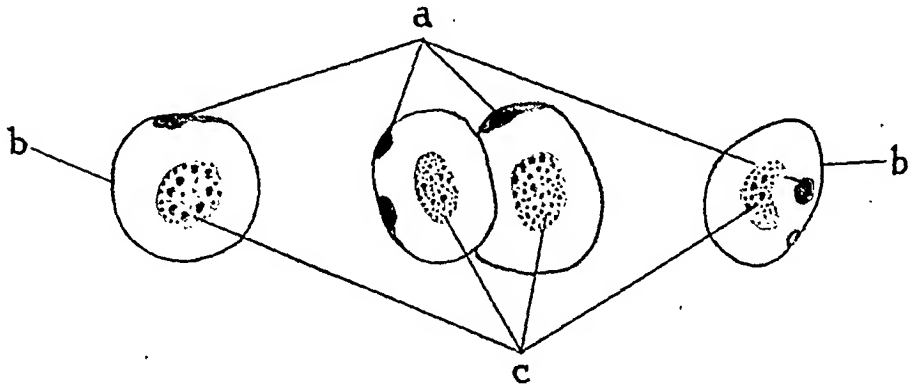
Liver, Kidney, and Heart.—In practically all of our cases we found in the liver more or less marked infiltrations with round cells and eosinophilic leucocytes, and sometimes proliferations around blood vessels and gall ducts. The kidneys exhibit in a few cases small hemorrhages and infiltration with round cells without significant changes in the glomeruli, convoluted and collecting tubules. In a few cases the same type of infiltration is encountered in the interstitial tissue of the heart

muscle. It is impossible to establish any relationship between these lesions and the activity of the virus of infectious laryngotracheitis, because lesions of the same kind occur in the kidneys and heart of "normal" chickens.

The remaining organs are free from lesions.

Intranuclear Inclusions in Epithelial Cells of the Respiratory Tract

In early stages of infectious laryngotracheitis characteristic intranuclear inclusions and nuclear changes could be demonstrated in the epithelial cells lining the mucous membrane as well as in those of the mucous glands of the trachea, and less frequently in those of the nasal



TEXT-FIG. 1. A drawing of four nuclei of tracheal epithelial cells. (a) Nucleoli; (b) nuclear membrane; (c) intranuclear inclusions showing argentophilic granules of various sizes and arrangement. Klarfeld's tannic acid and silver impregnation.

passages (9). They appeared to be characteristic of the disease. In some instances smaller acidophilic bodies were shown in otherwise unchanged nuclei, but usually the nuclei showing the typical changes exhibited a more or less marked enlargement and hyperchromasia of the nuclear membrane, which caused it to appear sharply outlined. These inclusions, homogeneous in appearance, were usually situated centrally, and occupied a large part of the nucleus (Fig. 7, Text-fig. 1). They were usually round or oval, sometimes long and sausage-shaped, and occasionally had the form of a diplococcus. They were sharply outlined and took a pink stain with Giemsa's stain, while they stained

red with methylene blue-phloxin, hematoxylin-safranin, Goodpasture's fuchsin-methylene blue, and Gerlach's stain for Negri bodies. The nucleoli, of which these cells generally possessed one to three, were located peripherally, often attached to the nuclear membrane, and stained very intensely with basophilic stains. Another striking and characteristic feature was that the space between the nuclear membrane and the inclusion remained entirely unstained (Fig. 7, Text-fig. 1). They appeared as early as 12 hours after inoculation and more often occurred in all of a group of epithelial cells than in a single one. The protoplasm of the cells showing them stained very slightly, was more or less vacuolated, and the cell boundaries were no longer distinguishable (Fig. 7). The appearance and size of the intranuclear bodies as well as the changes in the nuclei bore a close resemblance to those described in herpes, varicella, virus III of rabbits, and submaxillary gland disease of guinea pigs (10). The precise character of the intranuclear acidophilic substance has not been ascertained. It did not stain with the fat stains or give the iron reaction, but it showed a slightly positive Feulgen reaction for thymonucleic acid (11). In using various silver stains numerous small argentophilic granules could be revealed inside the inclusions (Text-fig. 1). It is a striking fact that in many instances the affected nuclei underwent dissolution. We have not observed similar changes of epithelial cells in other diseases involving the same structures, such as A-avitaminosis, coryza contagiosa, and fowl-pox (12, 13). Since the same type of intranuclear inclusions and nuclear changes are present in other virus diseases, we feel justified in considering them a result of virus activity in the epithelial cells of the trachea and larynx.

DISCUSSION

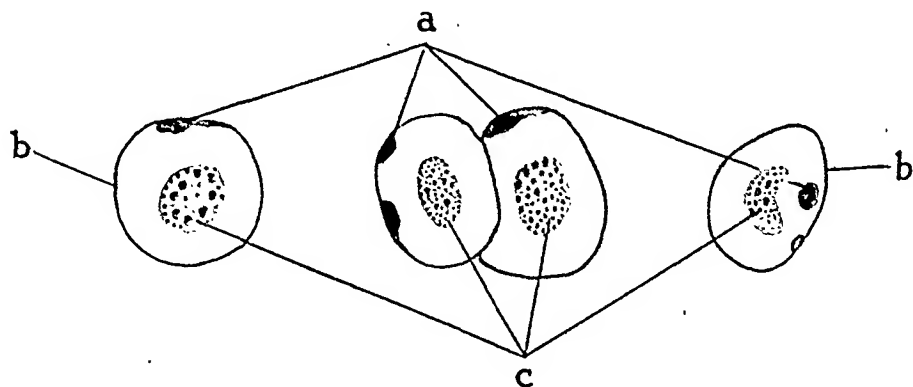
In spontaneous and experimentally produced cases of laryngotracheitis the lesions are most pronounced in the larynx and upper part of the trachea. Because the histological findings correlate with the clinical symptoms and the gross anatomical picture, we believe that the name "infectious laryngotracheitis," as used by Graham, is most truly descriptive and should therefore be adopted. Our findings indicate furthermore that the involvement of the nasal passages, communicating sinuses, and eyelids depends upon the mode of infection and the

muscle. It is impossible to establish any relationship between these lesions and the activity of the virus of infectious laryngotracheitis, because lesions of the same kind occur in the kidneys and heart of "normal" chickens.

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passages (9). They appeared to be characteristic of the disease. In some instances smaller acidophilic bodies were shown in otherwise unchanged nuclei, but usually the nuclei showing the typical changes exhibited a more or less marked enlargement and hyperchromasia of the nuclear membrane, which caused it to appear sharply outlined. These inclusions, homogeneous in appearance, were usually situated centrally, and occupied a large part of the nucleus (Fig. 7, Text-fig. 1). They were usually round or oval, sometimes long and sausage-shaped, and occasionally had the form of a diplococcus. They were sharply outlined and took a pink stain with Giemsa's stain, while they stained

red with methylene blue-phloxin, hematoxylin-safranin, Goodpasture's fuchsin-methylene blue, and Gerlach's stain for Negri bodies. The nucleoli, of which these cells generally possessed one to three, were located peripherally, often attached to the nuclear membrane, and stained very intensely with basophilic stains. Another striking and characteristic feature was that the space between the nuclear membrane and the inclusion remained entirely unstained (Fig. 7, Text-fig. 1). They appeared as early as 12 hours after inoculation and more often occurred in all of a group of epithelial cells than in a single one. The protoplasm of the cells showing them stained very slightly, was more or less vacuolated, and the cell boundaries were no longer distinguishable (Fig. 7). The appearance and size of the intranuclear bodies as well as the changes in the nuclei bore a close resemblance to those described in herpes, varicella, virus III of rabbits, and submaxillary gland disease of guinea pigs (10). The precise character of the intranuclear acidophilic substance has not been ascertained. It did not stain with the fat stains or give the iron reaction, but it showed a slightly positive Feulgen reaction for thymonucleic acid (11). In using various silver stains numerous small argentophilic granules could be revealed inside the inclusions (Text-fig. 1). It is a striking fact that in many instances the affected nuclei underwent dissolution. We have not observed similar changes of epithelial cells in other diseases involving the same structures, such as A-avitaminosis, coryza contagiosa, and fowl-pox (12, 13). Since the same type of intranuclear inclusions and nuclear changes are present in other virus diseases, we feel justified in considering them a result of virus activity in the epithelial cells of the trachea and larynx.

DISCUSSION

In spontaneous and experimentally produced cases of laryngotracheitis the lesions are most pronounced in the larynx and upper part of the trachea. Because the histological findings correlate with the clinical symptoms and the gross anatomical picture, we believe that the name "infectious laryngotracheitis," as used by Graham, is most truly descriptive and should therefore be adopted. Our findings indicate furthermore that the involvement of the nasal passages, communicating sinuses, and eyelids depends upon the mode of infection and the

course of the disease. The virus, which has thus far in Beach's investigations proven pathogenic for chickens only, seems to possess a distinct organ selectivity, being limited in its activity to the mucous membranes of the respiratory tract. Though it occasionally enters the blood stream it does not lead to characteristic lesions in other tissues.

As in many other virus diseases, the agent producing infectious laryngotracheitis causes an inflammatory process with edema or cellular infiltration, followed by retrogressive changes and a certain amount of proliferation. The course of the disease is so rapid that it is extremely difficult to determine precisely the location and the character of the primary injury and to follow the development of the lesions and the order of their occurrence. This difficulty is rendered still greater by the complexity of the mucous membrane of the respiratory tract. By a careful study of cases killed 6, 12, and 24 hours after intratracheal inoculation with a small dose of virus, it has been found that lesions of the surface epithelium may be present without notable changes in the submucosa. Films made with the mucus covering the mucous membrane of the trachea in these cases show a great many desquamated epithelial cells with various kinds of degeneration. Red blood corpuscles are also found in films from the mucous membrane of these cases. The conclusion seems justified that the virus attacks the epithelial cells first, penetrating afterwards to the submucosa. The inflammatory process occurring in this layer is of such severity as to constitute the predominating phenomenon. The destruction taking place later in the disease can be attributed not only to the virus but to mechanical pressure from edema, accumulations of infiltrating cells, or hemorrhages; and occasionally to the presence of secondary invaders.

The finding of intranuclear inclusions resembling those in herpes, varicella, virus III of rabbits, and submaxillary gland disease of guinea pigs, adds another disease to those in which such forms are associated with viruses. In laryngotracheitis they are present only in the earlier stages of the disease, which may be considered evidence in favor of their relationship to the virus.

SUMMARY AND CONCLUSIONS

1. The characteristic lesions of infectious laryngotracheitis are ordinarily restricted to the respiratory tract and are most pronounced in

the larynx and trachea. Sometimes the eyelids are affected. A certain percentage of the cases are associated with bronchitis and peribronchitis, pneumonic areas and hemorrhages in the lung, while the involvement of the nasal passages, communicating sinuses, and eyes seems to be dependent upon the mode of infection and the course of the disease.

2. The virus affects the epithelial cells primarily, but soon inflammation develops in the submucosa and underlying parts. Edema is often extremely pronounced in the submucosa. The destruction taking place at later stages is due to edema, cellular infiltration, and hemorrhages, and in some instances to secondarily invading bacteria.

3. Characteristic intranuclear inclusions in the epithelial cells of the trachea are present in many cases. They bear a close resemblance to the inclusions occurring in herpes, varicella, virus III of rabbits, and submaxillary gland disease of guinea pigs.

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EXPLANATION OF PLATES

PLATE 58

FIG. 1. Cross-section through trachea of a chicken killed $2\frac{1}{2}$ days after intratracheal inoculation. Infiltration of the submucosa and mucosa, thickening of epithelial layers, and proliferation and degeneration of cells at the surface. Hematoxylin-eosin. $\times 136$.

FIG. 2. Cross-section through trachea of a chicken killed $3\frac{1}{2}$ days after intratracheal inoculation. Destruction of the surface epithelium, marked edema and slight infiltration of the entire mucous membrane, separation and destruction of glands. Hematoxylin-eosin. $\times 136$.

FIG. 3. Cross-section through larynx of a spontaneous case. Round cell infiltration and enormous thickening of the mucous membrane. Destruction of the surface epithelium and that of the glands. Hematoxylin-eosin. $\times 136$.

PLATE 59

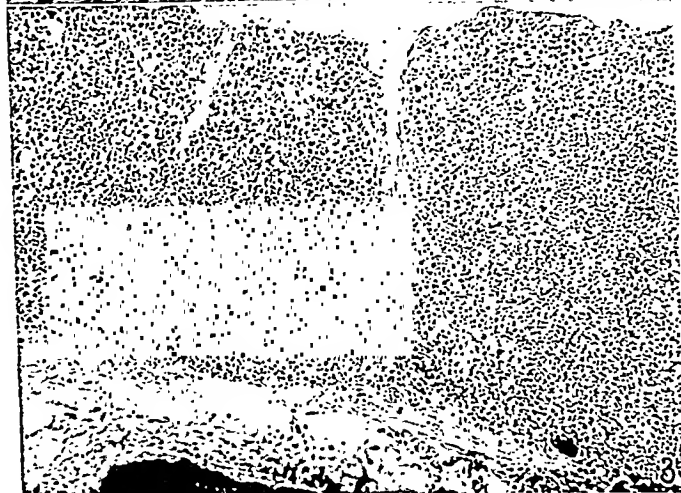
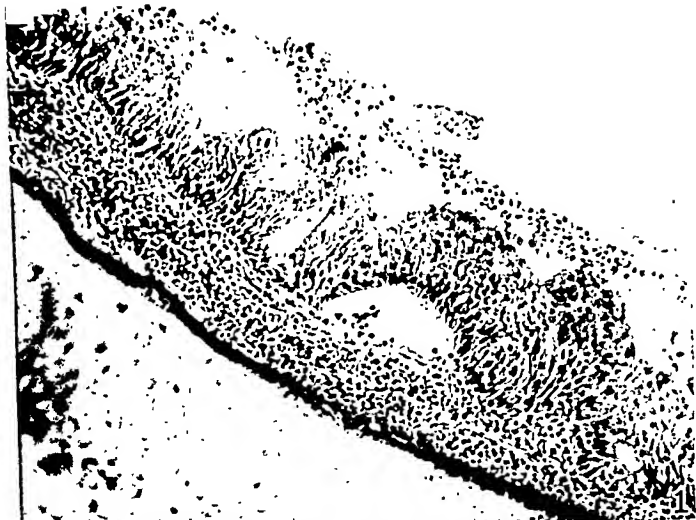
FIG. 4. Lung from chicken dead 6 days after intratracheal inoculation. Bronchitis and edema. Hematoxylin-eosin. $\times 180$.

FIG. 5. Necrotic hemorrhagic focus in the lung walled off by the formation of fibroblasts and giant cells. Chicken dead of spontaneous disease. Hematoxylin-eosin. $\times 184$.

PLATE 60

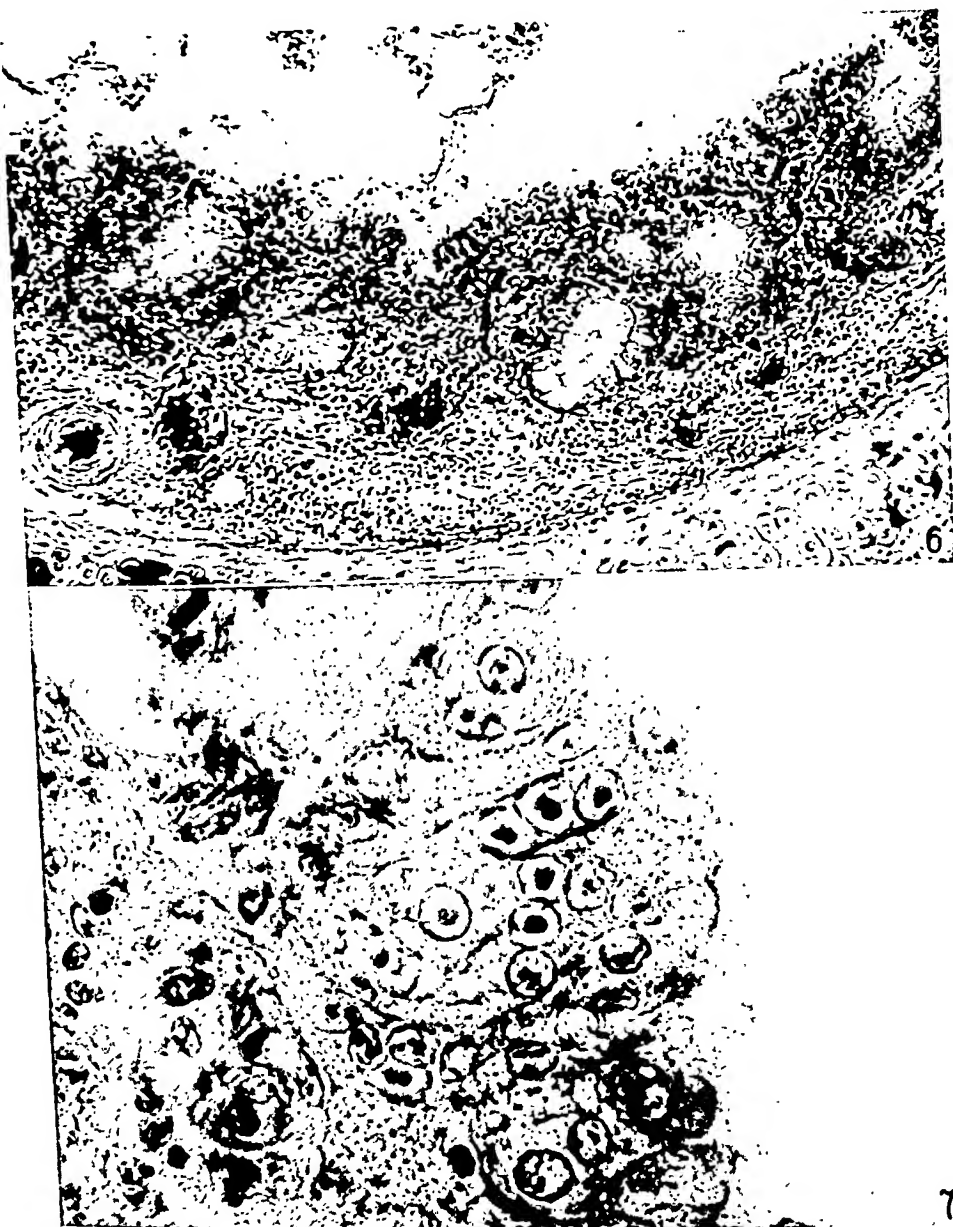
FIG. 6. Turbinate. 4 days after inoculation into cleft palate. Inflammatory process of the nasal mucosa. Hematoxylin-eosin. $\times 184$.

FIG. 7. Group of tracheal epithelial cells from a spontaneous case. A large proportion of the nuclei contain characteristic inclusions. In some of such nuclei the nucleoli are attached to the nuclear membrane. Giemsa stain. $\times 1000$.



(Seifried: Histopathology of laryngotracheitis)





(Seifried: Histopathology of laryngotracheitis)

ACTION OF METHYLENE BLUE ON BODY TEMPERATURE AND METABOLISM*

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From the results of numerous investigators who used methylene blue in the study of the nature of cell combustions we know at present that this dye can accomplish or increase oxidations in various biological systems.

Thunberg (1) and Ahlgren (2) observed the reduction of methylene blue by washed muscle to which were added a great variety of organic substances, and they explained this reduction by the discharge of "activated hydrogen" from the substrates. Later on, von Szent-Györgyi (3) and Fleisch (4) independently showed that the reduced dye can give up this hydrogen to molecular oxygen and thereby establish "the bridge between activated hydrogen and oxygen."

The mechanism of biological oxidations with methylene blue has been explained quite differently by Warburg (5). From the study of the oxygen consumption of red blood cells treated with methylene blue he concluded that the dye probably acts by bringing the oxidizing agent proper, which is an iron compound, to a higher level of oxidation, in which state only, it can and does oxidize the substrate present.

Methylene blue can be supposed to act, by whatever mechanism, by keeping one of the factors of normal cell oxidation in a higher state of activity. The question then arises whether this factor can be induced to greater activity in a normal cell. In other words, can methylene blue increase the oxygen consumption and metabolism of normal cells, tissues or organisms?

W. Palladin and his coworkers (6) studying the effect of methylene blue on the respiratory exchange of plants, found that the CO_2 production of etiolated stems of *Vicia faba* was increased up to 107 per cent when they had been colored by methylene blue. The observed stimulation was less with stems of *Pisum sativum* and almost negligible in the case of seeds.

In 1921 C. Heymans and E. Maigre (7, 8) found that the injection of methylene blue intravenously in dogs was followed by a marked rise in body temperature,

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and the appearance of all signs of a typical hyperthermia. Further experiments by C. Heymans and J. F. Heymans (9) showed that the hyperthermia was not due to a retention of heat but was accompanied by an increase in metabolism. The CO_2 and the calorie output were both raised to three or four times their normal value. The dose used in most experiments was about 50 mg. per kilo of body weight and was usually divided over a series of successive injections. Neither convulsions nor any muscular phenomena were observed which could explain the rise in metabolism.

The hyperthermizing action of methylene blue was further studied by J. Dadelez and W. Koskowski (10) who state that they found, after injection of methylene blue in dogs, an increased glycogenolysis in the liver and glycogen deposition in the muscles. Their explanation of the hyperthermia as a consequence of the increased sugar output from the liver is not supported by sufficient evidence.

A. Mayer and G. Nichita (11) confirmed C. and J. F. Heymans' results. C. Heymans showed thionine and methylene azure to be also hyperthermizing with dogs.

The hyperthermizing action of methylene blue is, however, not general. While it occurs in dogs and monkeys (*Macacus*), results on rabbits and guinea pigs have been negative (12). Similarly, A. D. Stammers (13) could not detect any variation in the body temperature of rats injected with methylene blue. He probably injected subcutaneously.

The modifications in oxygen consumption of intact cells and tissues under the influence of methylene blue were described by Barron and Harrop (14, 15). It was found that the addition of methylene blue increased the oxygen uptake of sea urchin and starfish eggs and also of mammalian non-nucleated erythrocytes. The effect is less marked with leucocytes and with avian nucleated erythrocytes but with the latter the oxygen consumption still went up to two or three times its basal value. Passing on to the study of normal tissues, Barron (16) showed that methylene blue did not increase their oxygen consumption but rather depressed it, the only exceptions being the spleen and the gray matter of the brain. Methylene blue increased the oxygen uptake of tumor tissue. Summarizing these results, Barron could state the rule that only cells or tissues which have an aerobic glycolysis can be induced to a higher rate of metabolism by means of methylene blue.

How are the findings on entire animals and on tissues to be reconciled? It might not be a mere coincidence that Barron used tissues taken from the rat and that the rodents, *i.e.*, the rat and the rabbit, according to the authors quoted above do not show any methylene blue effect on body temperature. C. and J. F. Heymans as well as A. Mayer and G. Nichita suggested the possibility that the dye might act in the dog as an oxidation catalyst. The former authors, however, rather believed in an action on the nervous system. Methylene blue is an inhibitor of the parasympathetic system and, according to H. Meyer (17), such a pharmacological behavior goes along with the capacity to raise metabolism. In the light of Barron's rule, it should be possible to bring some evidence in favor of one of these views. If it is possible to induce a rise in metabolism in the

rat by injecting methylene blue the logical conclusion would be that the hyperthermia is not due to direct action on the cell. If no rise in metabolism can be produced in the rat, the rise occurring in the dog would take on a new significance, which would make desirable further study of isolated dog tissues.

The experiments here reported show that methylene blue injected intravenously in sufficient doses in the white rat may cause either a rise or a fall of body temperature, and may or may not bring about a transitory rise of total metabolism, depending chiefly on the external temperature. By the argument above stated, it may be concluded that this effect of methylene blue is probably not the result of direct catalytic action of the dye upon cellular oxidation, but is due to indirect disturbance of thermoregulation, and, doubtless, of other functions of the organism.

Description of the Experiments

The white rats used as experimental animals were not under 150 gm. of body weight and fasted for 24 hours. Three different doses of methylene blue were used, a single injection being given each animal. The controls received 1 cc. per 100 gm. of body weight of 0.72 per cent NaCl solution. The experimental animals received the same relative amount of NaCl solution in which was dissolved 0.5, 0.4 or 0.25 per cent of methylene blue. These concentrations corresponded to 50, 40 and 25 mg. of dye per kilo of body weight. The solution was made up a few minutes before the injection by dissolving the methylene blue through a small filter paper by pouring on warm NaCl solution and making up to volume. Before injection the temperature of solution was brought to 38°C. The dye used was a sample, U.S.P. medicinal, from the National Aniline and Chemical Co., and was recrystallized once from water.

Rectal temperatures were taken using a small certified rectal thermometer, pushed in always to the same point and held in place for 3 minutes.

In studying the influence of methylene blue on the respiratory exchange the general procedure was as follows. The animal was lightly anesthetized with ether and the femoral vein exposed. The incision was then closed with wound clips. At least 1 hour afterwards the rat was subjected to a measurement of its respiratory exchange by the Haldane method (18) over a period of 2 hours. This period is referred to as "Period I." Next, the animal was fastened in dorsal position to a board, the clips were removed and methylene blue was injected slowly through a fine needle into the femoral vein. The incision having been closed again, the respiratory exchange was measured over another 2 hour period, mentioned in the tables as "Period II." Sometimes the metabolism was determined over a third period.

The respiratory chamber was a 1150 cc. bottle with a wide neck closed tightly by a rubber stopper carrying in- and outlet tubes and a thermometer. Both tubes were provided with stop-cocks of 4 mm. bore. The bottle was lying on the side thus forming a chamber of long shape in which the animal rested on a wire screen.

The chamber was aerated with laboratory air, through suction by means of a vacuum pump. An air flow of about 150 l. per hour was drawn through the apparatus. In the ingoing as well as in the outgoing air, CO_2 was absorbed over soda lime (Wilson) and water over pumice stone soaked in sulfuric acid according to Haldane (18). The absorption bottles and the chamber containing the animal were carefully weighed to 1 cg.

The chamber with the animal was weighed after a "preliminary ventilation" of 10 minutes for the first period, *i.e.*, the one preceding the injection of methylene blue. The preliminary ventilation was sometimes longer before the period following the injection as will be explained below.

Measuring the respiratory exchange before the injection or after injection of 0.72 per cent NaCl solution offered no difficulty. The chamber was very easily kept at the desired temperature. In experiments at 30°C . and above, and on cold days in experiments at 28° and 29.5°C ., intermittent heating with an electric bulb covered with tin-foil had to be resorted to. In the period preceding the injection, the temperature could be kept constant within less than 1° .

After the injection of methylene blue, however, several rather unusual difficulties arise, which we have thought necessary to describe in some detail.

The injection of dye is followed by a state of marked weakness lasting 5 to 10 minutes. When the animal is already in the respiratory chamber under preliminary aeration, a period of restlessness sets in, of variable degree and duration. Sometimes there will be only a few rather violent efforts to get out of the chamber or the animal will change position frequently. There are no convulsions but only very brisk general movements. The animal has a tendency to lie down with its face resting at the points where the air current is strongest, namely, at the ventilating devices to shield it from the draft. At the same time, but only with a high dose of dye, a marked salivation sets in, respiration is accelerated and deep. Later on respiration will still be rapid but more shallow. The period of activity lasts for a variable length of time, from 10 minutes to $\frac{1}{2}$ hour. The animal then quiets down and remains so. In many cases delay is caused in the start of the metabolism period following the injection and one has to leave out a certain period of time during which the respiratory exchange would obviously be altered by the exaggerated muscular activity.

We usually weighed the animal chamber after 10 or 15 minutes of preliminary ventilation and assumed that the error introduced by a few movements of the animal would be lost in the measurement of the metabolism over the next 2 hours. When this assumption was not permissible on account of continuous or prolonged

agitation, the chamber was closed, when the activity began to subside, and weighed again. A new absorption train was connected with it and the metabolism taken over 2 hours, counting from the new starting point. In cases in which the amount of time lost is large, e.g., $\frac{1}{2}$ hour, the values for the metabolism do not represent the immediate effect of the dye. Low values are obtained since the action of the dye is transient. Muscular activity also may cause delay in weighing.

The second difficulty is to maintain the constancy of temperature in the chamber. This temperature has a tendency to rise while the animal chamber is being weighed. When the chamber was interposed in the ventilation circuit the aeration, together with a current of air directed upon it, quickly lowered the temperature, which could then be considered constant, except for a few minutes following the weighing.

In some cases, however, it was not possible to cool the chamber to the temperature of the period before the injection. The metabolism period was then continued at this higher temperature which in some experiments differs from the preceding one as shown in the tables. Inspection of the variation in metabolism obtained in the second period shows, however, that this difference cannot be accounted for by the difference in environmental temperature alone. The reader is referred to the studies on metabolism of the white rat by K. Goto (19), and lately by F. G. Benedict and G. McLeod (20). For temperatures increasing above 28°C., the former found an average increase in metabolism of 5.1 per cent per degree. Benedict and McLeod found the metabolism above 28°C. to be essentially constant. Goto's measurements go up to 33°C., Benedict and McLeod's up to 31.7°C.

No error resulted from a difference in temperature at the beginning and at the end of a metabolism period, except in cases in which the chamber, after the methylene blue injection, could not be cooled, as explained above. With our chamber the error thus introduced is of the order of 0.5 mg. for a difference of 1°, below the accuracy attained in the weighings. It has not been taken into account.

The last point to be considered is the variation of the water vapor tension in the metabolism chamber. After the animal has been injected with methylene blue, as a feature of the hyperthermic reaction there occurs a large increase in water output either through the lungs or from the vaporization of saliva. At a certain moment the water vapor condenses on the walls of the chamber and in the outlet tube. This condensation can hardly be prevented by increasing the rate of ventilation unless beyond the rate at which absorption of CO₂ and water is complete. This increased water tension causes an error in determining the loss of weight of the chamber which is very difficult to estimate for every single experiment. It occurs only in experiments where 50 mg. of methylene blue per kilo are injected and in which the increase in metabolism is high. An upper limit of the error can be obtained by calculating the volume of water vapor present in the chamber, knowing its temperature and the tension of water vapor at this temperature. The water drives out an equal volume of air and the weight of the latter is the error

introduced in the final weighing of the chamber. To give an example, in Experiment IV, reported in Table I, the error would be maximal and the figure for oxygen consumption in Period II about 6 per cent too low.

But the error is certainly less since the air after the preliminary ventilation is not quite dry and because it is doubtful whether it still becomes saturated at the end of the metabolism period when the animal's reaction has to a large extent subsided. Finally, the correction would apply only to experiments where 50 mg. of methylene blue per kilo were injected and when the injection was followed by a high rise in metabolism.

For these reasons we have not introduced any correction on account of water vapor. In cases showing a high increase in metabolism, the oxygen consumption after injection of methylene blue is probably somewhat greater than indicated by the figures.

The Haldane method was used as described above in the experiments conducted at 28°C. or above. For the experiments performed at 23.5°C. the technique was modified. They were carried out in the early summer and it was necessary to immerse the chamber in ice water. The chamber was larger and provided with an inner lining of cardboard to prevent direct contact of the animal with the cold wall. The wire screen on which the animal rested was likewise replaced by cardboard. The absorption train was as usual but only CO₂ output was determined, the correctness of the absorption being checked by weighing the control bottles. Measurements were performed on two series of animals, one injected with methylene blue, the other with NaCl solution, after they had been kept in the chamber for 1 hour, in order to allow the metabolism to reach a stable level.

The Action of Methylene Blue on the Body Temperature and the Respiratory Exchange of the White Rat

The effect of methylene blue on the respiratory exchange and body temperature is shown in the tables. Figures for oxygen consumption and CO₂ production represent grams per hour and per kilo of body weight.

Table I gives the results obtained with 50 mg. of dye per kilo, for temperatures from 28° to 31.5°C. It can be seen that the increase in metabolism noted after the injection is very variable but that, the higher the temperature of the animal chamber, the more marked is the effect. This is best brought out by comparing the experiments conducted at 28°C. with those at 31.5°C. The average increase in O₂ consumption in Experiments V, VII and VIII (at 28°) is 16.3 per cent, whereas the same average of Experiments IV, X and XI (at 31.5°) is 54.5 per cent. The individual variations are very marked

and the per cent increase in metabolism does not seem to be closely related either to the initial level or to the temperature to which the animal is exposed.

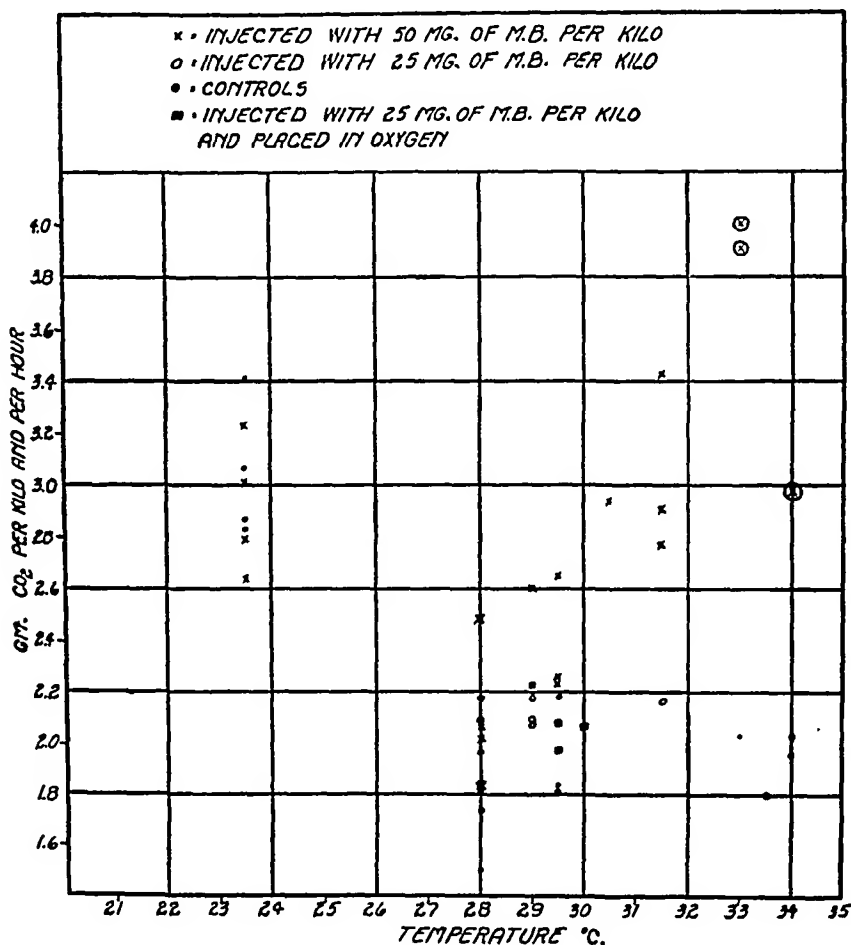


CHART 1. Showing CO₂ output at various environmental temperatures

Table II shows some results obtained with doses of 40 and 25 mg. of dye per kilo. The effect of the 40 mg. dose is likewise dependent on the environmental temperature. Injections of 25 mg. per kilo have but little effect which does not increase with temperature.

TABLE II

Experiment No.	Sex and body weight	Respiratory exchange			Temperature of respiratory chamber	Rectal temperature B. = before A. = after	Change in respiratory exchange		
		I (Before M.B.)	II	III			II	III	

A. Experiments with 40 mg. of M.B. per kilo									
XII	m., 178	O ₂ : 2.30	2.58		I : 27	B. II:38.2	O ₂ : +12		
		CO ₂ :2.33	2.50		II :28	A. II:37.2	CO ₂ : +7.3		
		RQ:0.735	0.705						
XIII	m., 178	O ₂ : 1.94	2.41		I : 27	B. II:36.7	O ₂ : 24.4		
		CO ₂ :1.91	2.39		II :27	A. II:35.9	CO ₂ : 25.1		
		RQ:0.716	0.718						
XIV	f., 194	O ₂ : 1.60	2.76		I :30	B. II:38.6	O ₂ : 66.3		
		CO ₂ :1.52	2.83		II :30	A. II:38.9	CO ₂ : 81.0		
		RQ :0.692	0.747						

B. Experiments with 25 mg. of M.B. per kilo									
XV	f., 205	O ₂ : 1.68	2.00		I :31.5	B. II:38.6	O ₂ : +19		
		CO ₂ :1.66	2.17		II :31.5	A. II:38.9	CO ₂ : +30.7		
		RQ:0.717	0.79						
XVI	m., 188	O ₂ : 1.85	2.25		I :29.5		O ₂ : +21.6		
		CO ₂ :1.81	2.19		II :29.5		CO ₂ : +21.0		
		RQ:0.71	0.705						
XVII	m., 184	O ₂ : 2.03	2.17		I :29		O ₂ : +5.4		
		CO ₂ :1.99	2.08		II :29		CO ₂ : +4.5		
		RQ:0.71	0.695						
XVIII	m., 210	O ₂ : 1.85	1.85	1.77	I :28		O ₂ : 0		-4.3
		CO ₂ :1.78	1.81	1.78	II :28		CO ₂ : +1.7		0
		RQ:0.70	0.71	0.72	III:28				

XIX	m., 160	O ₂ : 2.12 CO ₂ : 2.00 RQ: 0.684	2.25 2.09 0.677	I : 28 II : 28	B. II: 37.1 A. II: 36.6	O ₂ : +6.1 CO ₂ : +4.5
XX	m., 172	O ₂ : 2.18 CO ₂ : 2.12 RQ: 0.708	2.27 2.18 0.70	I : 28 II : 28	B. II: 38.1 A. II: 36.1	O ₂ : +4.1 CO ₂ : +5.4
XXI	m., 169	O ₂ : 2.01 CO ₂ : 2.04 RQ: 0.738	2.21 2.18 0.718	I : 29 II : 29	B. II: 38.2 A. II: 36.8	O ₂ : +9.9 CO ₂ : +6.9
XXII	m., 182	O ₂ : 1.84 CO ₂ : 1.84 RQ: 0.727	2.14 2.09 0.708	I : 29 II : 29	B. II: 37.7 A. II: 36.4	O ₂ : +16.3 CO ₂ : +13.6

Experiment No.	Sex and body weight	Respiratory exchange			Temperature of respiratory chamber °C.	Rectal temperature B. = before A. = after °C.	Change in respiratory exchange	
		I (Before M.T.)	II	III			II	III
C I	f., 166 gm.	O ₂ : 2.02 CO ₂ : 1.97 RQ: 0.71	1.99 1.97 0.73		I : 28 II : 28		O ₂ : -1.5 CO ₂ : 0	
C II	m., 186	O ₂ : 1.53 CO ₂ : 1.55 RQ: 0.737	1.57 1.51 0.70	1.53 1.57 0.75	I : 28 II : 28 III: 28		O ₂ : +2.6 CO ₂ : -2.6	0 + 1.3
C III	f., 181	O ₂ : 1.89 CO ₂ : 1.93 RQ: 0.74	1.83 1.81 0.736		I : 28 II : 28		O ₂ : -3.2 CO ₂ : -6.2	
C IV	m., 171	O ₂ : 1.75 CO ₂ : 1.78 RQ: 0.74	1.84 1.84 0.727	2.02 1.89 0.685	I : 29.5 II : 29.5 III: 29.5	B. II : 37.9 B. III: 37.8 A. III: 37.9	O ₂ : +5.1 CO ₂ : +3.4	+15.4 +6.2
C V	m., 170	O ₂ : 1.91 CO ₂ : 1.88 RQ: 0.716	1.91 1.82 0.694	1.85 1.91 0.75	I : 29.5 II : 29.5 III: 30	B. II : 37.3 B. III: 37 A. III: 36.6	O ₂ : 0 CO ₂ : -3.2	-3.1 +1.6
C VI	m., 221	O ₂ : 1.68 CO ₂ : 1.66 RQ: 0.718	1.79 1.74 0.71	1.77 1.70 0.70	I : 28 II : 28 III: 28	B. II : 37.7 B. III: 37.8 A. III: 38.1	O ₂ : +6.5 CO ₂ : +4.8	+5.4 +2.4
C VII	m., 179	O ₂ : 1.90 CO ₂ : 1.82 RQ: 0.695	2.01 2.04 0.737		I : 31 II : 33	B. II : 37.5 A. II : 37.8	O ₂ : +5.8 CO ₂ : +12.2	
C VIII	m., 214	O ₂ : 1.94 CO ₂ : 1.94 RQ: 0.727	2.10 2.03 0.704		I : 34 II : 34	B. II : 38.9 A. II : 38.3	O ₂ : +8.2 CO ₂ : +4.6	
C IX	f., 247	O ₂ : 1.84 CO ₂ : 1.88 RQ: 0.742	1.80 1.80 0.727		I : 33.5 II : 33.5	B. II : 39 A. II : 39.5	O ₂ : -2.2 CO ₂ : -2.8	
C X	f., 186	O ₂ : 2.07 CO ₂ : 2.02 RQ: 0.708	1.99 1.96 0.717		I : 34 II : 34	B. II : 39.5 A. II : 39.5	O ₂ : -3.9 CO ₂ : -2.9	

Table III gives the figures for the controls. It can be seen that the injection of NaCl solution has no significant effect.

The significance of the results appears clearer when only the absolute values for O₂ uptake and CO₂ production before and after the injection are considered. In Chart 1, the values for CO₂ output obtained in the experiments with 50 and 25 mg. of methylene blue per kilo have been plotted against the chamber temperature at which the measurement was made. Only figures for "second periods" have been plotted to picture the behavior of the controls.

TABLE IV
*Experiments at 23.5°C.
Dose of Methylene Blue: 50 Mg. per Kilo*

Sex	Body weight	Transition period	CO ₂ per kilo and per hr.	Rectal temperature	
				Before	After
A. Controls					
	gm.	hrs.	gm.	°C.	°C.
f.	218	1	3.07	36.9	37.0
m.	244	1	2.87	36.4	36.6
m.	226	1	2.83	36.7	36.7
m.	170	1	3.41	36.9	37.8
B. Injected with methylene blue					
f.	170	1	3.23	36.9	34.0
f.	212	1	2.62	36.3	—
m.	186	1	2.79	36.7	35.0
m.	234	1	2.31	37.1	32.8

There is a definite relation between the temperature and the absolute level which the metabolism reaches after the injection. The active dose of methylene blue is 50 mg. per kilo. At 28°C. or above, it raises the average respiratory exchange, and the more so, the higher the temperature.

The three experiments conducted above 32°C. with the high dose of methylene blue gave results indicated on Chart 1 by crosses surrounded by circles. The rise in metabolism is quite high in these cases. It must be said, however, that the measurements only extended over a

period of about 30 minutes. At these temperatures the injection of the dye is rapidly fatal. Since we cannot strictly compare these results with the others they have not been included in Table I.

The results obtained at 23.5°C. are given in Table IV and have also been represented on Chart 1. At this temperature there is no hyper-metabolizing effect from the dye injection. Methylene blue rather

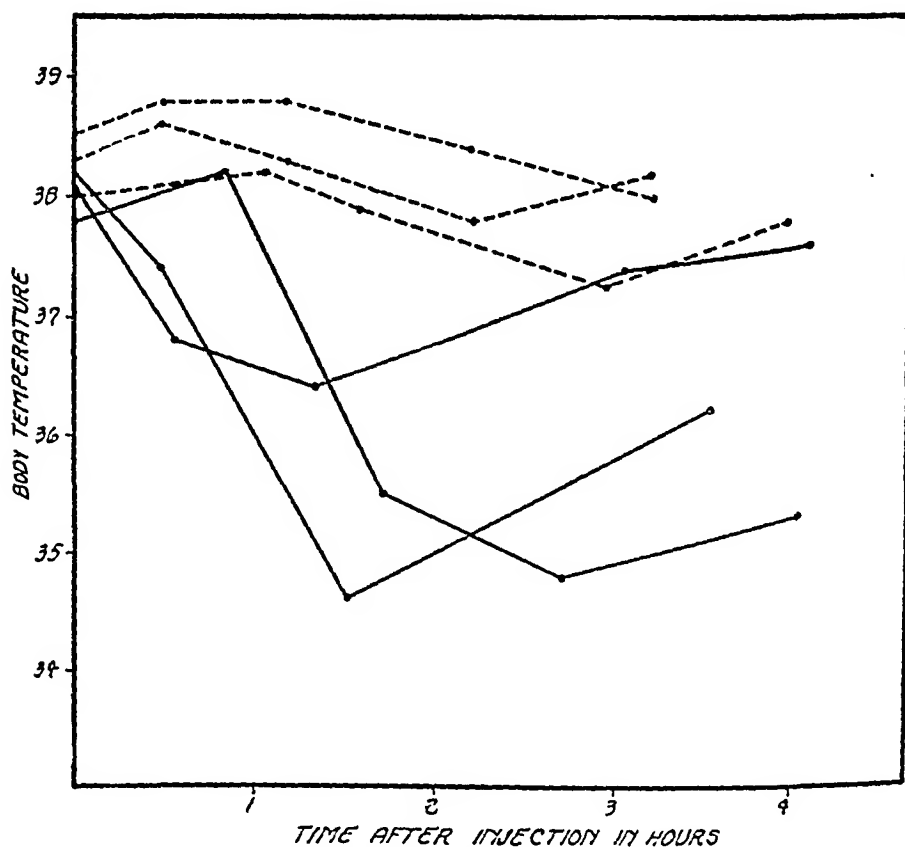


CHART 2

seems to depress the metabolism of the experimental animals as compared with the controls.

The tables also give the variations of the body temperature. Typical results are obtained with the higher dose of dye. It can be seen that at higher temperature it is hyperthermizing but at 29.5°C. and below, it constantly causes a fall in body temperature. To illustrate the hypothermizing action of methylene blue in high doses at "room

temperature" a few temperature curves are given in Chart 2 referring to animals which were injected with 50 mg. of methylene blue per kilo and kept in the laboratory, the room temperature varying from 26° to 24°C.

Experiments with Methylene Blue and Oxygen

It is shown by the experiments of J. A. Campbell (21) that, when breathing oxygen at a partial pressure higher than in air, the oxygen tension in the tissues is increased. Hence, there was a possibility that the hypermetabolizing action of methylene blue could be enhanced by oxygen at high tension. It may be recalled, however, that high oxygen tensions have been shown not to modify the metabolism of vertebrates. This is agreed upon by several investigators. (Quoted in (22).)

In our experiments the metabolism was taken in the way described except that oxygen from a tank was made to pass through the apparatus at a pressure slightly above atmospheric, and at the usual rate. The animal chamber was weighed after a preliminary ventilation of 10 minutes before, and again at the end of each period; *i.e.*, it was always weighed filled with oxygen. The absorption bottles were aerated for 15 minutes with CO₂ and H₂O-free air before the final weighings. Hence, they were weighed filled with air. Blank trials showed that without animal the different parts of the apparatus did not vary in weight. The animals were subjected to a first measurement in oxygen, then injected with the dye (or with NaCl solution, if they were controls) and again their metabolism was taken in oxygen.

The results on injection of 50 mg. of dye per kilo were inconsistent. Those obtained with 25 mg. per kilo show clearly that the level of metabolism induced by methylene blue is not raised by oxygen. The values for CO₂ output after administration of the 25 mg. dose have been indicated on Chart 1.

The experiments led to a curious observation. It is long known that breathing pure oxygen is fatal to vertebrates, usually in several days, at least with larger animals. The cause of death is pulmonary congestion and edema. References to this point are found in Binger *et al.* (22).

When rats were kept in the chamber with a sufficient stream of oxygen going through, to which some moisture had been added, they

were found to be still alive after 24 hours, while animals injected with methylene blue invariably died in about 10 hours. Lorrain Smith (23) states the time in which pure oxygen at 1.3 atmosphere pressure killed mice as varying around 60 hours.

Postmortem examination showed incompletely collapsed lungs which seemed edematous and showed extensive red areas, very different from the normal yellowish pink color. Our thanks are due to Dr. H. A. McCordock, who examined the organs histologically.

Two animals were injected with methylene blue and one of them was kept in oxygen. This animal showed a widespread pulmonary edema. The alveoli were filled with coagulated albuminous fluid. The capillaries of the alveolar walls were congested, and the lymphatic vessels of the interstitial tissue dilated and filled with edematous material. There were no changes in the other organs. The control animal showed no pulmonary edema. The capillaries were congested. The adventitia of many blood vessels appeared swollen and edematous.¹

Edema and consolidation of the lungs caused death in animals exposed to high oxygen tension in Lorrain Smith's experiments. Apparently this process is speeded up considerably by methylene blue.

DISCUSSION

From the results obtained it may be concluded that, at least in the white rat, methylene blue is either hyperthermizing or hypothermizing, all depending on the temperature of the environment.

The dye is not very hyperthermizing. Only in the experiments conducted at 30.5°C. and higher did a rise in body temperature occur which, with the exception of one case (Experiment IX of Table I), did not exceed 0.6°C. Practically, it may be said that 30.5° to 31.5°C. is the range of outside temperature at which methylene blue does not cause a significant change in body temperature. In an environment above 32.5°C., the rise in body temperature is very rapid and high, and death of the animal occurs in about $\frac{1}{2}$ hour. As has been said before, these experiments have not been included in Table I. It may be said here that at the moment they died the rats were found to have a rectal temperature of 41.1°C., and in one case 41.6°C.

¹ The terms of this description are taken from Dr. McCordock's report.

The hyperthermia caused by methylene blue is, therefore, by no means as marked as that which Heymans and Maigre observed in dogs. On the contrary, methylene blue at 29.5°C. and below is definitely hypothermizing.

The control animals also showed variations in body temperature which is conceivable since the temperature regulation in the white rat is by no means efficient. The action of methylene blue on the body temperature of the white rat may, therefore, best be stated as follows: While a normal animal, after having stabilized its body temperature at a level depending on the environmental temperature, is able to maintain this level, an animal injected with an effective dose of methylene blue is unable to do so, outside of a very small interval situated around 31°C.

The variation in body temperature depends, of course, on the balance between heat production and heat loss, the latter being accomplished in the rat through radiation and thermal polypnea. Methylene blue, therefore, must act upon the mechanisms of heat loss regardless of the metabolism level. At 28°C., respiration is accelerated and emission of water vapor is increased beyond the actual need. At lower temperature (23.5°C.), methylene blue does not interfere much with the compensatory heat production. Respiration is accelerated for a short time. Whether any increase in water output occurs, we have not been able to ascertain but it seems quite probable. It must be said that at this temperature the action of the dye is much more transient, and this probably explains why, in these cases, the fall in body temperature is not as marked as might be expected from what is observed at 28°C.

We have no evidence that the increase in metabolism caused by methylene blue is due to increased carbohydrate oxidation. No significant changes in the respiratory quotient occurred and in this respect our results differ from those obtained by Dadlez and Koskowski (24) who observed with dogs respiratory quotients close to unity after methylene blue injection.

Whether methylene blue acts as an oxidation catalyst in the cells cannot be wholly excluded but the dependence of its action upon the environmental temperature is definitely against this view.

The experiments at the higher temperatures show that in an injected

animal conditions are given which allow a higher metabolism and that no factors, as for instance limitation of oxygen supply, can come into play. The latter point is strengthened by the negative results obtained when the animals were subjected to oxygen, at least with low doses of dye, in which pulmonary complications proceed sufficiently slowly.

SUMMARY

1. Methylene blue injected intravenously in white rats is hyperthermizing or hypothermizing according to the environmental temperature.

2. It causes an increase in metabolism at 28°C. or above. At lower temperature it does not affect, or rather depresses, metabolism.

3. It does not seem likely that its hypermetabolic action is due to catalysis of cell oxidations.

4. In animals exposed to an atmosphere with a high partial pressure of oxygen, methylene blue causes pulmonary edema, much more rapidly than does oxygen alone.

The writer wishes to express to Dr. P. A. Shaffer his appreciation of the kind advice given him throughout this work.

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THE DEVELOPMENT AND LOCALIZATION OF THE DERMAL PNEUMOCOCCIC LESION IN THE RABBIT

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Although many studies have been made of the pathogenesis of human lobar pneumonia, little is known of the factors involved in the rapid development of the pneumonic lesion or of the conditions which bring about lobar localization. It is difficult to determine the mechanism by observations in clinical cases or in cases of experimental pneumonia in animals since under these circumstances the evolution of the lesions is at no time visible. In order to gain a clearer conception of what occurs during the infective process in man, an experimental study of the dermal pneumococcus infection in the rabbit was undertaken. This experimental disease has been described in previous communications (1-3) and has been shown to have many clinical, pathological, and immunological similarities to lobar pneumonia. Moreover, the site of the principal lesion is superficial and the progression of events may easily be followed. Although it is obvious that factors which are significant in this experimental disease may be of little importance in the pathogenesis of lobar pneumonia, it is possible that some of the experimental findings may aid in the interpretation of clinical data.

Histological studies have shown that the pronounced edema is the outstanding feature of the early lesion of the dermal pneumococcus infection in the rabbit (3). It develops before any significant change takes place in the cellular picture. In the spreading lesion the movement of the edema fluid through the tissues precedes the appearance of any other sign of reaction between the tissue and the bacteria. It has been shown by cultures that the advancing fluid carried with it the infecting organisms. In consequence the infection spreads not by an active invasion by the bacteria but by their passive transport and inoculation.

In view of the close relationship between the occurrence of the edema fluid and the infection, it seemed desirable to study the character of the fluid and the factors which influence its development, movement, and eventual localization.

The methods employed in the following experiments were essentially the same as those used in the studies previously reported (1). Rabbits were inoculated intradermally, at a site 8 cm. dorsal to the ventral midline, with 0.2 cc. of an 18 hour blood broth culture of Type I Pneumococcus. The results of the study of the characteristic lesion, as it develops under varying conditions, form the material of this report.

Properties of the Edema Fluid

It was necessary first to devise a means of obtaining the fluid in appreciable amounts. Aspiration was unsuitable for securing large quantities. Drainage from a small puncture wound proved more satisfactory. This is a slow and rather awkward procedure but by it as much as 5 to 10 cc. of edema fluid can be obtained from a single animal. Rabbits which have been infected intradermally 15 to 20 hours previously and in which the lesion is not markedly consolidated are most suitable. The skin over the dependent portion of the lesion is washed with alcohol and a sterile 18-gauge hypodermic needle inserted through the superficial skin layers at the point of greatest swelling. The animal is then held on a small elevated platform and the fluid seeping from the puncture wound is collected directly into a flask.

The edema fluid is slightly turbid and is of a light straw color. If there has been but little trauma and the fluid contains no blood cells, it does not coagulate on standing, even after many hours. Since it contains viable pneumococci and serves as an excellent culture medium for these organisms, it is the practice to pass the fluid immediately through a Berkefeld V filter.

This edema fluid not only fails to clot but also has the property of retarding the clotting of normal rabbit blood. The inhibition of coagulation of normal blood implies a definite increase in antithrombic property. That there is no deficiency in fibrinogen is shown by the fact that a firm clot forms promptly in the edema fluid on the addition of a small amount of "thromboplastin." It would appear that the failure of the edema fluid to coagulate is related to a disturbed balance between thromboplastic and antithrombic substances.

A study of the coagulation of the blood of rabbits during the course of the dermal pneumococcus infection shows that as the disease progresses the clotting time is increasingly prolonged, although, except in the most severe cases, the blood never fails to clot. In comparison with the clotting time of the edema fluid, the coagulation time of the blood withdrawn at 15 to 20 hours after infection is only slightly prolonged. Obviously the antithrombic substance is in greater concentration in the edema fluid than in the circulating blood. This fact would point to its origin in the lesion and subsequent diffusion throughout the body. If it originates in the lesion it is possible that it is derived directly from the infecting organism, or it may be formed by the interaction of the organism and the host.

The first of these possibilities, namely, that the antithrombic substance is derived from the pneumococci, is the more easily approached. The first material of bacterial origin to be examined was the specific capsular polysaccharide (Type I). The concentration of this material in the edema fluid was determined by precipitin titrations to be not greater than 1 part per 100,000 parts of fluid. Purified specific soluble substance in this and in greater concentrations was tested for its ability to retard the clotting of normal rabbit blood, but in no instance did it have any such effect. On the other hand, it was found that the addition of pneumococcus broth cultures to normal blood did influence the coagulation time, the intensity of this action depending upon the age of the culture employed. A culture several days old inhibited coagulation to a greater degree than did a young culture. This suggested that the inhibiting action might be due to the products of bacterial autolysis. Autolysates of pneumococci were therefore prepared and their action on coagulation studied. The washed organisms were suspended in physiological saline solution containing 0.5 per cent phenol. This suspension of bacteria was kept at 37°C. for 24 hours and then allowed to stand in the ice box for a week to permit autolysis to take place. After complete dissolution of the organisms the supernatant fluid was found to possess a marked anticoagulant action. In contrast to this the supernatant fluid of a similar suspension of washed bacteria which had been immediately heated at 80°C. for 15 minutes to prevent autolysis, hastened the coagulation of normal blood.

The effect of the various preparations on blood coagulation is shown in an experiment summarized in Table I. In this instance the control blood to which only saline solution had been added clotted in $3\frac{1}{2}$ minutes. Blood containing edema fluid required $8\frac{1}{2}$ minutes to coagulate, a period over twice as long. The pneumococcus autolysate prolonged the clotting time to 25 minutes, whereas an unautolyzed suspension of pneumococci containing an equivalent amount of bacterial material shortened the time to $2\frac{1}{2}$ minutes. The substance in the autolysate responsible for the anticoagulant action is heat-stable. It may be heated to 100°C . for 15 minutes without loss of activity.

It would seem, therefore, although this is not yet conclusively demonstrated, that the autolytic products of pneumococci are responsible for the anticoagulant action of the edema fluid. A further investigation as to the origin and chemical nature of the antithrombic sub-

TABLE I
Effect of Various Substances on Blood Coagulation

Substance	Coagulation time
	min.
Blood + saline.....	$3\frac{1}{2}$
Blood + edema fluid.....	$8\frac{1}{2}$
Blood + pneumococcus autolysate.....	25
Blood + pneumococcus autolysate previously heated at 100°C . for 15 min.....	25
Blood + suspension of washed heat-killed pneumococci.....	$2\frac{1}{2}$

stance is now in progress. In its chemical behavior it appears to be identical or closely related to the purpura-producing substance also present in pneumococcus autolysates (4). This substance is probably responsible for the increased vascular permeability in the infected area.

Dochez (5) observed that in acute lobar pneumonia the clotting time of the patient's blood is considerably prolonged. His evidence pointed to an increase in an antithrombic element and he suggested that some intoxicating substance originating from the infecting organism might have a specific effect on the liver and cause an increased production of antithrombin. The evidence herein reported, however, strongly suggests that the antithrombic substance is derived from the bacteria themselves. It is not unreasonable to suspect that some of the less well protected organisms are damaged in the earlier phases of the

pneumococcus infection, and that the resulting death of these cells allows autolysis to take place with the liberation of an end product which, by retarding coagulation and altering vascular permeability, promotes invasion by the uninjured organisms.

The antithrombic substance may play a significant rôle in the development of the lesion both in lobar pneumonia and in the experimental dermal infection, and it may well be that the production of this substance by pneumococci enhances the invasiveness of the organisms and accounts for the characteristic widespread edematous lesion.

The Direction of Spread of the Edema Fluid and Its Final Localization

In the many experimental infections of this type which we have observed, the lesion has invariably progressed toward the more dependent tissues. It seemed likely that the direction of progression is dependent upon gravity, but in order to establish this point it was necessary to rule out the possibility that the direction of spread might be related to the anatomical distribution of lymph and blood vessels in the involved area.

A study was made, therefore, of the anatomical distribution of the vessels in the flank area. The blood vessels were demonstrated by the application of xylol. The lymphatics were rendered visible by injections of India ink by the well known method used by Dr. Sabin. The general course of blood and lymph drainage in the skin of the flank area was found to be toward the axilla. The skin is rich in lymphatic anastomoses but in no instance did ink particles injected into the skin pass in the general direction taken by the progressing lesion, but instead they tended toward the larger lymphatics which follow the course of the larger veins toward the axilla.

A number of experiments have been carried out with the purpose of demonstrating the importance of gravity in determining the direction of spread of the edema fluid.

A rabbit was infected on the flank in the usual manner and then placed in a tight fitting box which was tipped to an almost vertical angle, with the head of the animal up. It had been inoculated at the usual site. The lesion proceeded downward, that is to say toward the tail in this case, and finally involved the entire gluteal region. After 18 hours the animal was replaced in its normal position. This resulted in a prompt alteration in the direction in which the lesion extended.

Advancing into the tissue which was now dependent it reached the midventral area. Because of the circuitous course taken by the edema the skin area between the point of inoculation and the ventral involvement remained entirely free from infection up to the time of the animal's death at 28 hours, whereas ordinarily it would have been early involved.

This experiment shows not only that the direction of spread is downward, its course being determined by the position of the animal, but also indicates that the lesion spreads most readily from a non-indurated border. In this case the lesion progressed caudally and at 18 hours the only non-indurated edge was in the gluteal region. The further spread, again determined by gravity, took place from this region, not from that of original inoculation which at this time was definitely indurated. A second experiment also demonstrated the action of gravity and in this instance the possibility of spread by way of the lymph and blood vessels was eliminated.

The hair was removed from the left hind leg of a rabbit and an intradermal inoculation of pneumococci was made on the inner surface of the leg below the popliteal region. The lymph drainage of this area was shown by India ink injections to be upward. The infection, however, progressed downward toward the foot. The edema over the lower leg was small in amount, however, presumably because of the tenseness of the skin over the extremity.

The foregoing experiment suggested that the direction of the spread in the flank area might possibly be modified by means of a tissue barrier. Several experiments have been carried out in which a barrier of granulation tissue was produced by means of a thermal cautery.

A rabbit was anesthetized and a line 0.5 cm. wide was seared across the flank by means of thermal cautery. The line of scarification had a diagonal situation, extending from dorsal-posterior to ventral-anterior regions at an angle of about 40° to the horizontal. After 1 week the seared skin was covered by a firm scab bounded on each side by a narrow band of granulation tissue. At this time an intradermal inoculation of pneumococci was made at a point dorsal to the upper fourth of the line. The initial lesion developed as usual but instead of progressing ventrally the advance followed the upper border of the scar. When the ventral end of the line was reached the fluid spread to the dependent areas and a typical lesion resulted.

The fact that the direction of spread was influenced by a mechanical barrier suggested the possibility that the same effect might be obtained

with an immunological barrier. In a previous paper (1) we have reported that the progress of the lesion could be entirely blocked by intracutaneous infiltration with specific immune serum, provided the infiltration was in the area ventral to the point of inoculation. Experiments have been carried out to determine if the direction of movement of the edema fluid might be modified by an immunological barrier.

Intracutaneous infiltrations of antipneumococcic serum (Type I) were made along a diagonal line situated like that in the preceding experiment. The inoculation was made at a corresponding point. As a control the same procedure was carried out in a second rabbit, but with normal horse serum substituted for the antipneumococcic serum. In the control animal the edema fluid spread directly downward in the usual manner, but in the animal in which the infiltration was with immune serum the spreading lesion followed along the dorsal aspect of the line for some 5 cm. before crossing.

It is probable that the entire spread of the lesion could be demarcated with serum injections provided it were possible to maintain the serum at the points of inoculation.

These experiments indicate that the direction of spread of the edema fluid, and therefore of the infective process, is determined largely by gravity and is independent of the anatomical distribution of lymph and blood vessels. They suggest that gravity may be a significant factor in the spread and localization of the infectious process in human lobar pneumonia.

Conditions Which Determine the Extent or Magnitude of the Involvement

An attempt has been made to analyze the factors which determine the magnitude of the lesion, that is, the size of the area occupied and the amount of edema in the involved zone. Probably the most important factor is to be found in the character of the skin. That of the flank and abdomen is loose whereas that over the lower part of the leg is quite tightly drawn. In the first instance the lesion is markedly edematous whereas in the second the edema is much less.

When the inoculation is made on the flank, the extent of the lesion is apparently determined by the state of fullness of the abdomen.

In the normal, well fed animal the abdomen below the usual site of inoculation is quite dependent and the lesion seldom spreads very far along the ventral midline. In the case of more emaciated animals, however, the abdomen is usually more or less retracted. In this case the lesion invariably gravitates along the midline and in some instances has been observed to involve the entire ventral region. Even in the case of well nourished animals a late spread of the lesion not infrequently occurs and this may be due to the fact that during the course of the disease a considerable loss of weight and retraction of abdomen frequently occurs.

TABLE II

The Relation of Distance Travelled by the Edema Fluid to the Size of the Lesion and the Amount of Edema

Rabbit	Point of inoculation	Observations 24 hours after inoculation	
		Area of lesion	Edema
1	Ventral midline	4 x 4 cm.	Slight amount
2	On the flank at a point 8 cm. dorsal to the ventral midline	Extends from point of inoculation to ventral midline where involvement measures 6 x 6 cm.	Considerable amount at ventral midline
3	1 cm. lateral to the dorsal midline	Extends from point of inoculation to ventral midline where involved area measures 7 x 11 cm.	Huge accumulation of edema fluid at ventral midline

Chance observation suggested that the distance that the edema fluid can travel may be important as determining the extent of the lesion. In order to study this question rabbits were inoculated at various sites, and the extent of the subsequent lesion and the amount of edema were observed. One experiment is summarized in Table II.

In the experiment summarized in Table II the animal which was infected at the ventral midline developed only a small lesion having an area of 4 by 4 cm. with only a slight amount of edema. In contrast the animal which was infected near the dorsal midline developed a widespread lesion in the ventral area with a huge accumulation of edema fluid. The animal which received the inoculation midway on the flank developed a lesion intermediate in extent and magnitude to those just described.

Perhaps the best explanation for this result is that in the animal inoculated at the highest point, the greatest drainage area was present.

Variations in Rate of Development of the Lesion

Factors of two kinds have been found to influence the rate of development of the local lesion. Thus the development is retarded (*a*) by conditions which depress the general resistance and thus favor the development of a primary bacteriemia, or (*b*) by the presence of substances which shorten the coagulation time of the blood, whereas an acceleration in the rate of development has been observed when certain microorganisms are used as associative infective agents with *Pneumococcus*.

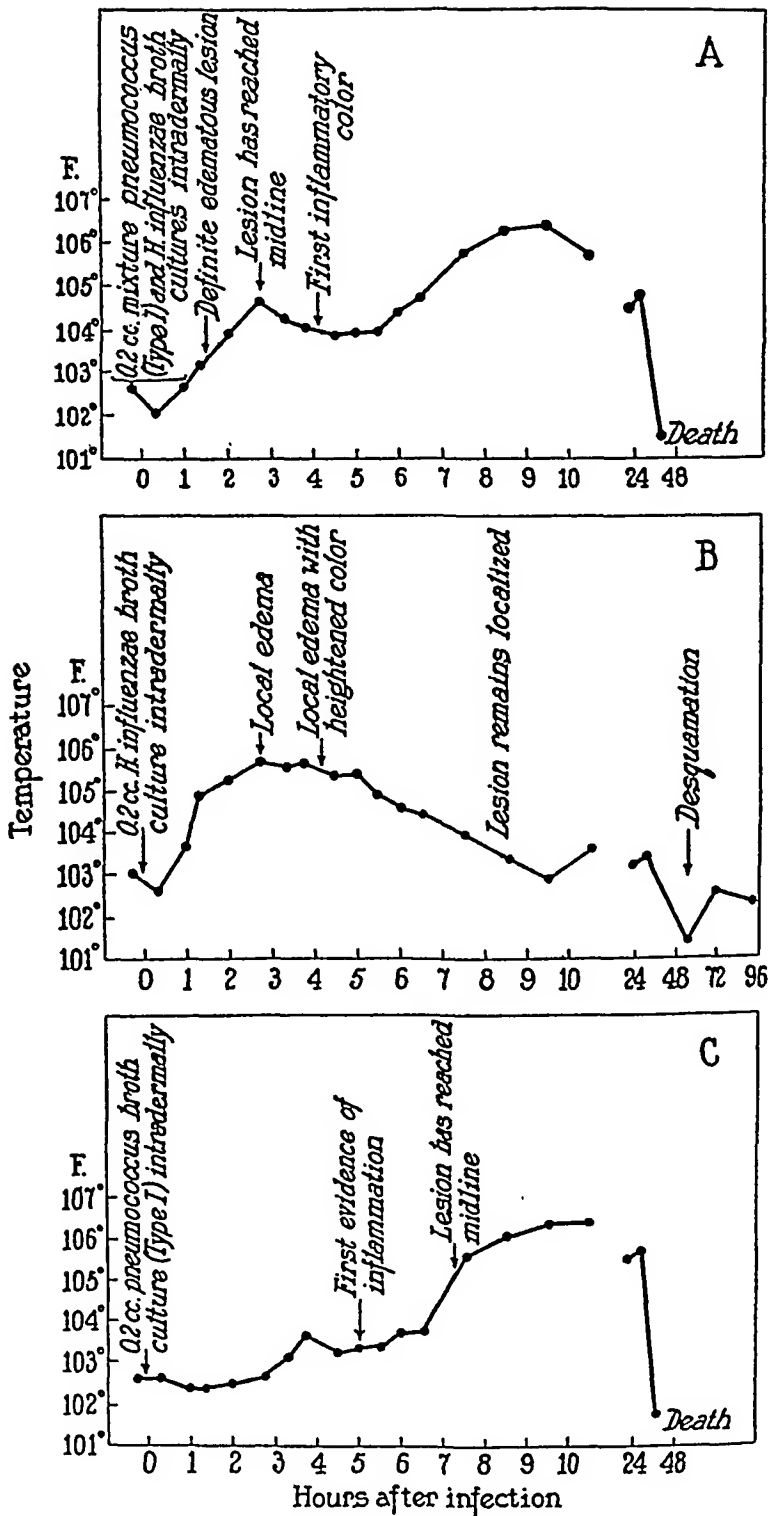
Some examples of depressing conditions may be cited. If a quantity of alcohol sufficient to produce intoxication is given intraperitoneally at the time of intracutaneous infection the body temperature is markedly depressed. The progress of the lesion is much the same as in the non-alcoholized animal but the disease is much more rapidly fatal and the animal dies before the ultimate local induration has developed. In this case the local infection is merely incidental to bacteriemia.

The same effect is obtained by placing the rabbit in an abnormal position after inoculation. Thus, if an infected rabbit is placed on a board in a dorsal recumbent position, the temperature is markedly depressed, and the resistance appears to be so low as to favor profound generalized infection and early death, before the development of any significant local infection.

An example of true retardation is obtained by injections of thromboplastin. Since the edema fluid coagulates *in vitro* if thromboplastin is added, it seemed probable that an analogous effect might occur *in vivo*. Injections of thromboplastin into normal animals showed that the effect on the coagulation time of the blood was more or less transient but fairly pronounced.

The local skin area of an infected animal was infiltrated with thromboplastin at the time when the lesion first showed signs of spreading. The ventral spread of the lesion was retarded for approximately 4 hours.

In a second experiment, repeated intravenous injections of thromboplastin were given, beginning at a time when the lesion showed the



TEXT-FIG. 1. Temperature charts of three rabbits infected intradermally with (A) *Pneumococcus* Type I and *H. influenzae*, (B) *H. influenzae*, (C) *Pneumococcus* Type I.

first evidence of ventral spread. The injections were repeated at approximately hourly intervals for 6 hours. During this time the progress of the lesion was almost entirely arrested. Within 2 hours after discontinuing the intravenous injections of thromoboplastin the edema fluid began to move ventrally at the usual rate.

An acceleration in the rate of development of the lesion has been observed when certain organisms are inoculated with the pneumococci. Apparently many Gram-negative organisms yield this result but it is best observed with *Hemophilus influenzae*. This is illustrated in Text-fig. 1, which gives the temperature charts of three animals, one infected with *Pneumococcus* Type I, one with *H. influenzae*, and one with a mixture of these two organisms. The comparative progress of the lesions is indicated.

H. influenzae, and especially recently isolated strains, if injected intracutaneously into the rabbit, give rise to a mildly inflamed edematous area which is indurated. This inflammatory reaction persists for 2 or 3 days. At the same time a transient thermal reaction occurs. The temperature rises abruptly after inoculation and reaches levels around 105°F. It begins to fall at about the 5th hour and has usually reached normal levels in from 7 to 8 hours.

When pneumococci alone are injected no inflammatory reaction or fever develops during the first 4 or 5 hours. Local inflammation then appears and the temperature begins to rise. The maximum temperature is usually not reached before the 7th or 8th hour and the edema fluid does not usually reach the ventral midline until about the 7th hour.

If an animal is infected with a mixture of these two cultures the progress of the lesion is strikingly different from that occurring when either organism alone is injected. Instead of remaining localized, the ventral extension begins quickly and the edema fluid may reach the ventral midline within 3 hours, or in less than one-half of the time elapsing when pneumococci alone are injected.

The mechanism of the acceleration in the case of the associative infection is not understood. This phenomenon may have some significance in human lobar pneumonia, for in the last 200 patients with lobar pneumonia in this Hospital, *H. influenzae* has been found to be present in the sputum in 72 per cent. It is possible therefore that this

organism may play a rôle in the rapid development of the lesion in lobar pneumonia.

SUMMARY

An attempt has been made to analyze the factors involved in the development and localization of the dermal pneumococcic lesion in the rabbit.

The character and quantity of the edema fluid which forms during the early phases of the lesion are intimately concerned in its development and spread. It contains an antithrombic substance, probably derived from the pneumococci, and delayed coagulation probably facilitates its movement through the tissue. The direction of spread in the skin is determined by gravity, and the fluid finally localizes in the more dependent regions. The distance that the pneumococcic lesion travels and the local tissue characters have much to do with the amount of fluid that accumulates.

Studies are also reported of some factors which alter the rate of spread of the edema fluid. An acceleration in rate occurs when *H. influenzae* is used as an associative infective agent with the *Pneumococcus*.

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STUDIES ON THE SPECIFIC CHARACTERISTICS OF SYPHILITIC BLOOD PROTEINS

I. SURFACE TENSION AND SOLUBILITY

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(Received for publication, August 25, 1931)

The object of the experiments here described, and of others to be reported later, has been to determine if possible wherein lies the difference between the composition of blood of human individuals having syphilitic infection and of normal blood of the species. The present paper, dealing with surface tension and solubility, aims to show that in some respects the physical properties of normal and syphilitic blood are at variance.

Probably the most extensive and most significant work on the surface-active properties of true and colloidal solutions in recent years has been reported by du Noüy. In 1918 he (1) devised a method for the determination of the surface tension of solutions which permits of very accurate measurements and which is rapid enough to allow large numbers of tests to be made without being excessively time-consuming. Also, repeated measurements may be made of the same surface after allowing time for changes and adjustment of the position of molecules in the surface layers and in the bulk of the liquid. The instrument used for making these determinations, "the du Noüy tensiometer," is of the type which measures the force necessary to break a platinum ring away from the solution under examination. Readings are made directly in dynes per centimeter on a scale which is graduated to 0.1 dyne. In 1923 the description of an improved tensiometer was reported (2).

Du Noüy (3) observed that there was a marked lowering of the surface tension of rabbit's blood serum upon standing undisturbed for a time, even in very high dilution. In another communication (4) he presents evidence to prove that at a dilution of 1:10,000 of blood serum a "monolayer" of oriented serum molecules is formed which reduces evaporation to a minimum, and also has to do with the lowering of the surface tension.

Ascoli (5) in 1910 measured the surface tension of immune serum and antigen, and of normal serum and antigen immediately and after 2 hours incubation. He concluded that the difference of the surface tensions could be used to determine the presence of antibody. Gouwens (6) disagreed with Ascoli on the reliability of

surface tension in differentiating antibody in serum, at least with *Bacillus paratyphosus* B as antigen. Along this same line du Noüy (7) observed that the time-drop (*i.e.* the difference in the surface tension readings made immediately after placing a 2 cc. sample of solution on a watch-glass so that it spreads to cover about 12 sq. cm. of surface and the readings taken 2 hours later) of immune serum was greater by 50 to 100 per cent than that of normal serum. He found the maximum drop still at a 1:10,000 dilution and drew the conclusion that the size of the micellae is probably unchanged in immune serum. The experiments here set forth tend to show that there may be a difference in size of the molecules of immune serum, basing the calculations on du Noüy's oriented layer theory and on results of solubility experiments.

Svedberg, in discussing a paper by Pauli (8), classed proteins as homogeneous with regard to the molecular weight, giving the figure 34,500 as the weight of the basic molecule and ovalbumin as an example of this class of proteins. It is then assumed that protein molecules of higher molecular weights, *i.e.* weights representing multiples of 34,500, are dissociated into molecules of a lower number of groups when the pH of the solution is sufficiently raised, until eventually with increasing alkalinity, all proteins have the same molecular weight, *viz.* 34,500. This theory is substantiated by the writer's experiments on surface tension determinations on normal and syphilitic human blood sera, and solubility experiments on separated globulins from these sera.

EXPERIMENTAL

In order to get an idea of the surface reaction of serum in general and to establish a basis for comparisons of subsequent determinations, it was considered well to get a number of measurements on the sera of several species of laboratory animals as well as to run some normal human specimens.

In the hope of finding out something of the character of the protective action of serum albumin in the precipitation reaction for syphilis in unheated sera, as referred to by Kurtz (9), it was decided to make examinations of heated portions of the specimens as well as unheated. The heating was done simply as in preparing to make a routine test for syphilis, *viz.* at 55°C. for 30 minutes.

Since the surface activity of solutions is so easily affected by the addition of foreign substances, all glassware used in handling solutions must be thoroughly cleansed immediately before use. Soaking for at least 18 hours in bichromate cleaning solution or boiling for $\frac{1}{2}$ hour in this solution is a good method, followed by rinsing in distilled water and drying immediately in the oven.

Determinations were made with a du Noüy tensiometer No. 10402 produced by the Central Scientific Company. Directions were followed closely in the operation of the instrument. Temperatures ranged in the neighborhood of 25°C., varying not more than 1° or 2° from that. No corrections were made for temperature change since the error is only a small fraction of a dyne per degree of change, and other factors may influence the readings more markedly. The unit of time is 1

hour. Du Noüy (10) pointed out that the curves at 1 and 2 hours were very little different in degree and were practically parallel. The diluent employed was physiologic sodium chloride, 0.85 per cent, having a surface tension of about 76 dynes which is but slightly or not at all affected by the time factor. Distilled water has a surface tension by this method of about 75.5 dynes and is little affected by the time factor.

Table I shows comparative measurements at various dilutions of the fresh sera from the normal rabbit, ox, sheep, guinea pig and human.

Curves were plotted for the values presented in Table I and are shown in Fig. 1.

TABLE I

Effect of Dilution on the Surface Tension of Normal Sera of the Human and Some Domesticated Animals

Dilution.....	Pure serum	1:100	1:1,000	1:10,000	1:50,000	1:100,000	1:250,000	1:500,000	1:1,000,000
	Surface tension in dynes								
Rabbit.....	58.4	66.1	70.5	76.1	75.9	75.8	—	74.9	74.1
Ox.....	61.3	66.3	68.8	75.9	74.5	—	76.2	—	75.0
Sheep.....	—	66.6	66.4	74.0	75.7	—	75.9	—	—
Guinea pig.....	—	66.0	64.9	74.8	75.5	74.7	—	75.3	75.3
Human.....	—	68.5	68.7	76.5	76.2	—	76.4	—	—

It is readily seen that in the freshly diluted serum, a dilution of 1:10,000 is sufficiently great that the solid matter does not change the surface tension appreciably from that of physiologic saline or distilled water.

However, in the normal specimen heated for $\frac{1}{2}$ hour at 55°C. the surface activity is modified in dilutions up to 1:10,000 as observed by du Noüy (11), indicating increased dissociation of the protein molecules at this temperature. In other words when the molecules are split up and become smaller due to the action of heat, the total surface exposed by the particles in a given amount of solution is increased so that the dilution must be increased in order to obtain a single layer at the surface which accounts for the maximum decrease in tension after a period of time. Also the measurements after an interval of 1 hour

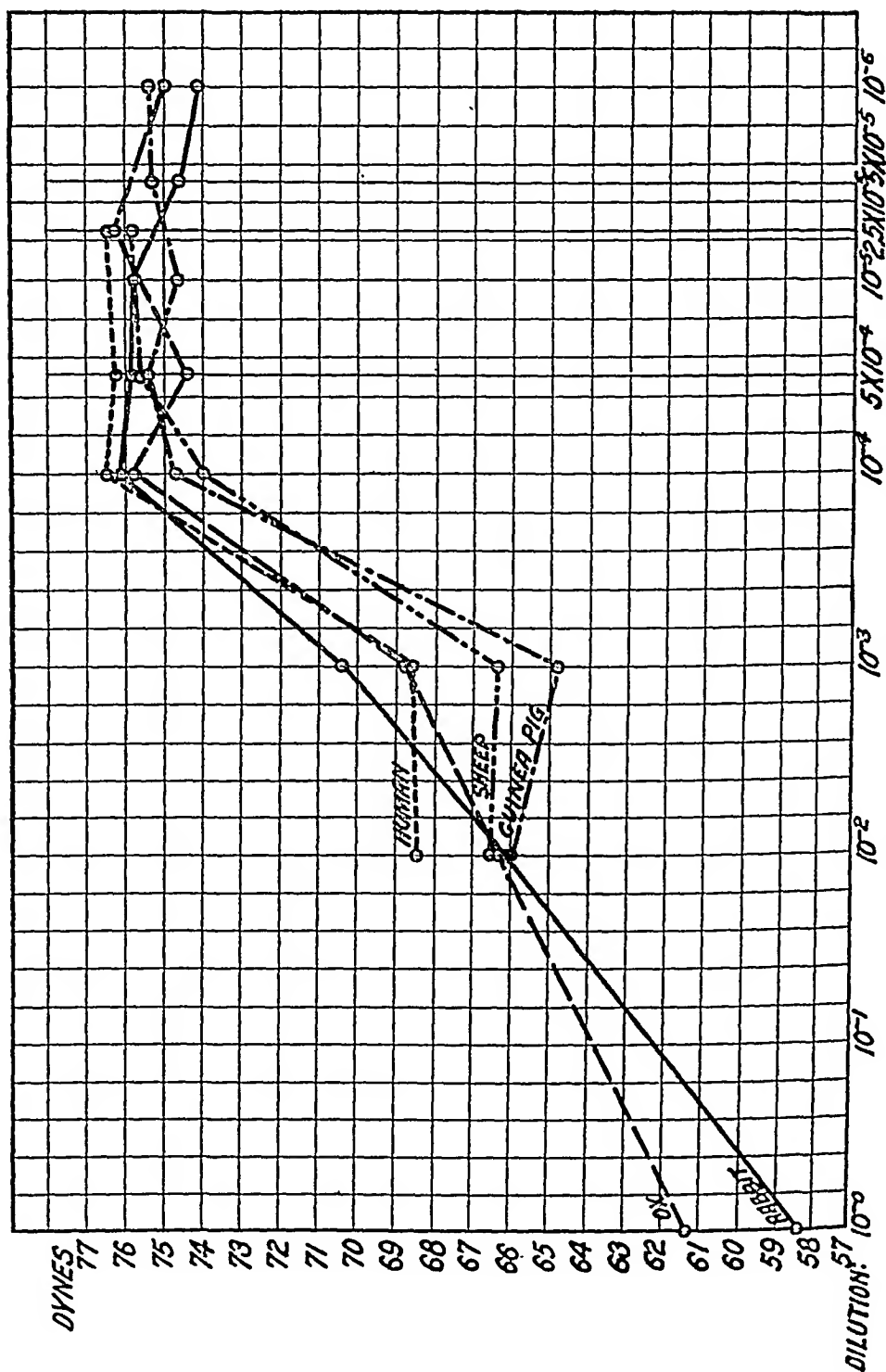


FIG. 1. Effect of dilution on the surface tension of normal sera

are modified in dilutions up to 1:50,000, thus giving a greater time-drop at this dilution.

The difference in the initial readings and those made after a 1 hour time interval, or the time-drop, of the raw and heated specimens may be better contrasted in graph form (Figs. 2, 3 and 4).

TABLE II

Initial Surface Tension Measurements of Normal Sera, Raw and Heated at 55°C. for $\frac{1}{2}$ Hour (See Figs. 2, 3 and 4)

Dilution.....	1:100	1:1,000	1:10,000	1:50,000	1:250,000
	Surface tension in dynes				
Human (raw).....	68.5	68.7	76.5	76.2	76.4
“ (heated).....	66.7	66.7	69.0	75.9	76.6
Rabbit (raw).....	—*	69.1	73.5	75.2	75.0
“ (heated).....	—	67.5	71.1	75.2	74.8
Guinea pig (raw).....	—	65.3	74.4	74.9	74.7
“ “ (heated).....	—	61.8	68.9	74.7	74.1

* 1:100 dilutions omitted because of low surface activity in this zone.

TABLE III

Measurements of Same Specimens after Standing Undisturbed for 1 Hour at Room Temperature of 25°C. (See Figs. 2, 3 and 4)

Dilution.....	1:100	1:1,000	1:10,000	1:50,000	1:250,000
	Surface tension in dynes				
Human (raw).....	61.5	64.1	66.2	67.1	76.0
“ (heated).....	64.0	63.4	61.6	63.2	75.1
Rabbit (raw).....	—	62.5	62.9	66.0	75.0
“ (heated).....	—	61.1	61.3	62.1	75.0
Guinea pig (raw).....	—	62.4	64.2	69.8	74.0
“ “ (heated).....	—	60.1	59.7	64.6	69.2

Here we see that the greatest distance between the initial curve and the 1 hour curve is at a dilution of 1:10,000 for the raw serum and at 1:50,000 for heated serum. In other words, the peak of the curve for heated normal serum is moved over toward the higher dilutions than that for raw serum. These values are not always at 10,000 and 50,000,

but may vary with different individuals between dilutions of 1:1,000 and 1:100,000. All observations made so far have fallen within these values, and the majority were in the neighborhood of the values in the cases illustrated.

Effects of Immunity on Surface Tension: Syphilis

Having made detailed explanation of the characteristic differences between raw and heated normal serum, a brief description to compare

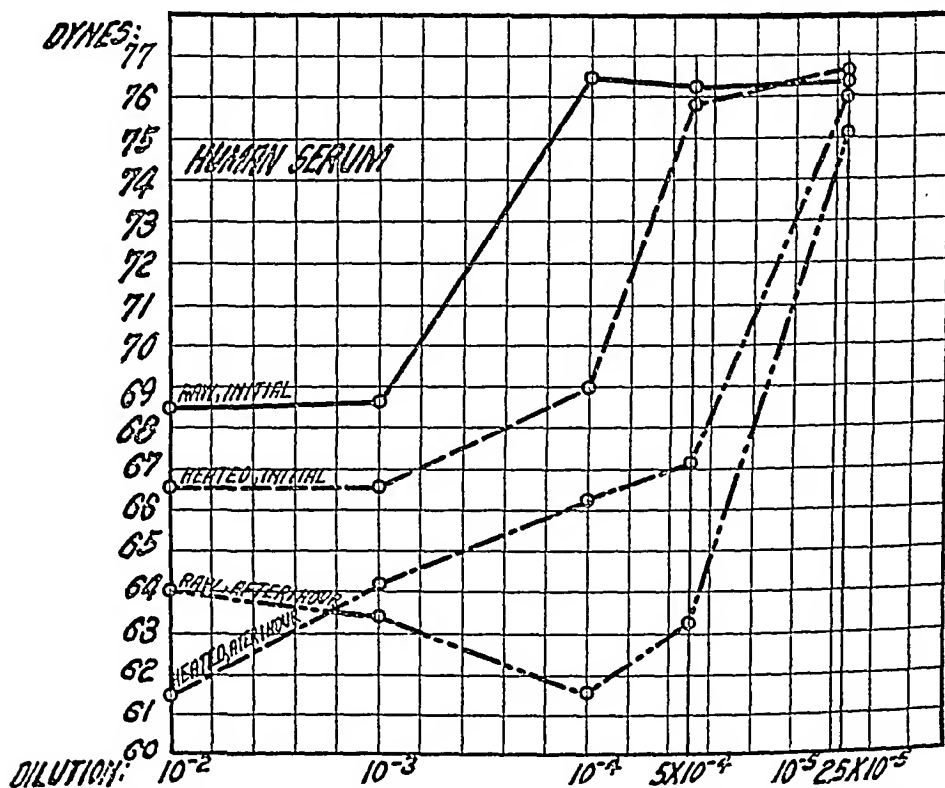


FIG. 2. Effect on surface tension of heating normal serum for $\frac{1}{2}$ hour at 55°C .

the values of similarly treated immune serum will suffice. For this purpose rabbit's blood immune to sheep's red blood corpuscles was used as a comparative example along with the blood from syphilitic human individuals.

Here the greatest time-drop comes at the same dilution for heated as for raw serum, or at a lower dilution as illustrated in Fig. 7. As in the case of normal sera, the greatest divergence of the curves does not

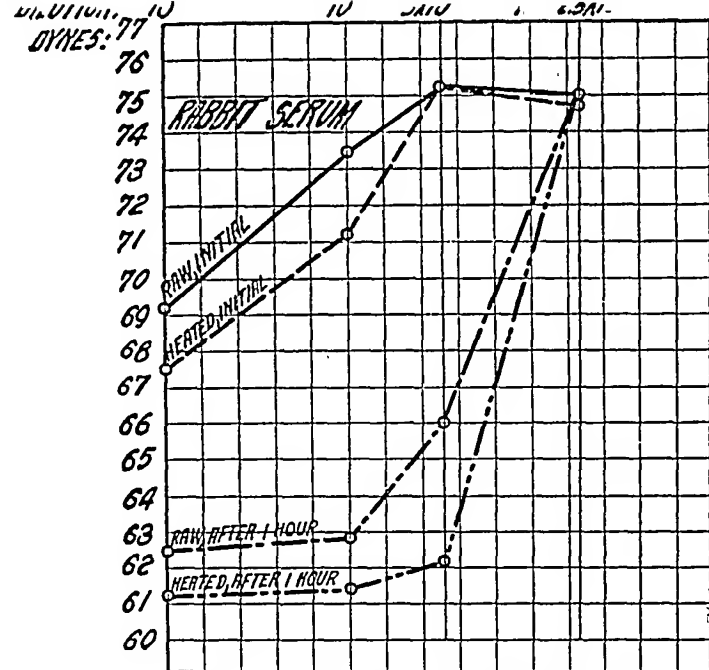


FIG. 3. Effect on surface tension of heating normal serum for $\frac{1}{2}$ hour at 55°C .

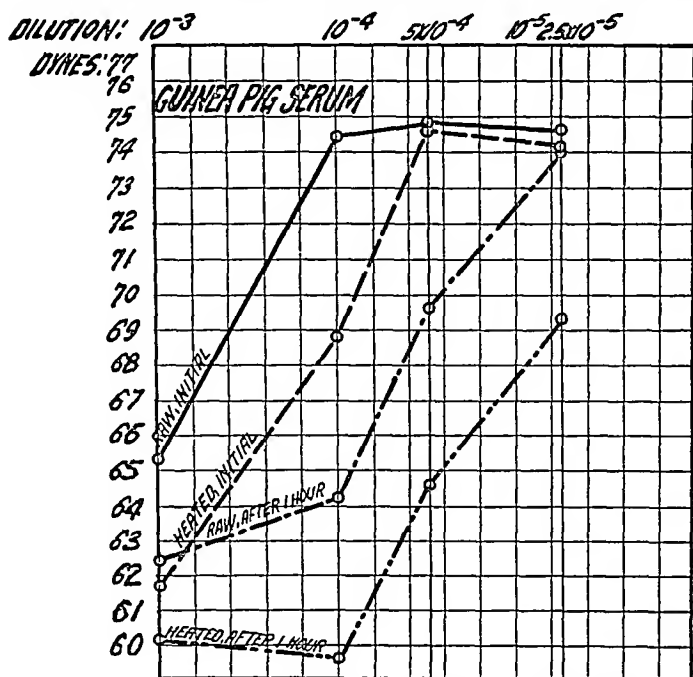


FIG. 4. Effect on surface tension of heating normal serum for $\frac{1}{2}$ hour at 55°C .

SYPHILITIC BLOOD PROTEINS. I

The solution was usually free of electrolytes in about 4 hours. Then the pension of globulin was divided into a number of tubes for centrifugalization, which the supernatant fluid was decanted and buffered solvent added to redissolve the sediment. A series of tubes of albumin suspension were also prepared to demonstrate the difference in solubility of the two main protein constituents of the serum. In the light of the recent findings of Svedberg's discussion of Pauli's paper (8) concerning molecular weights of proteins and their electrochemical behavior in regard to decomposition and reorganization, it seems as though more

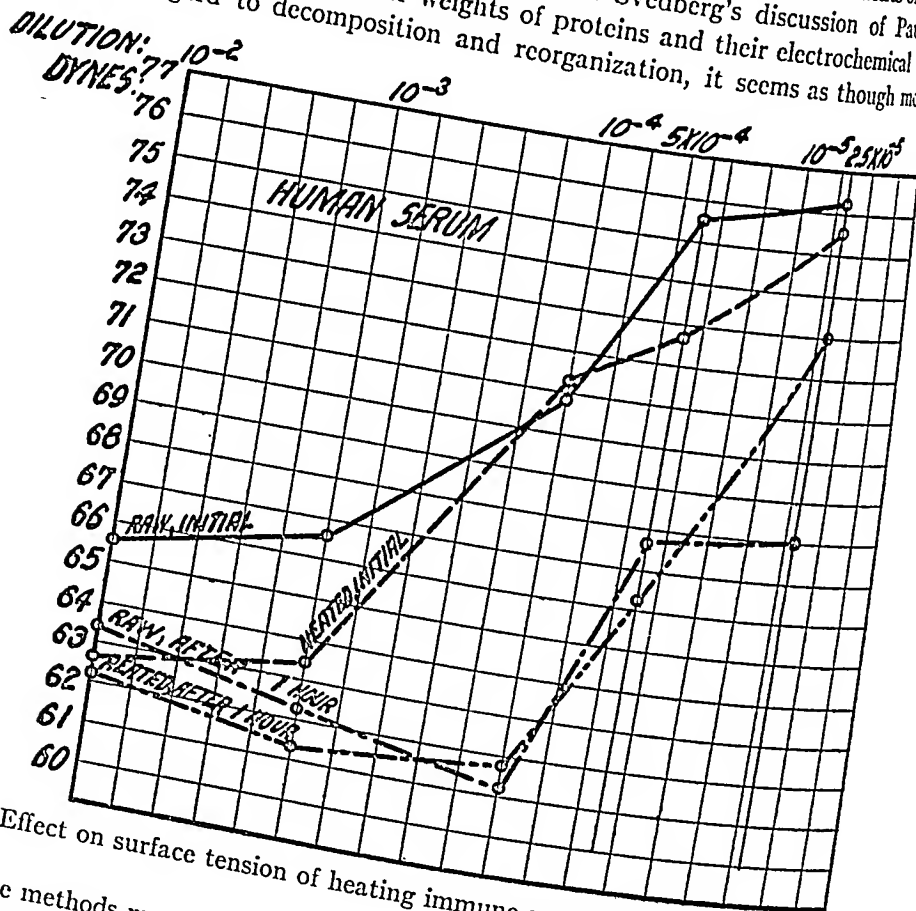


FIG. 6. Effect on surface tension of heating immune serum for $\frac{1}{2}$ hour at 55°C.

dependable methods may be worked out for diagnosis by precipitation and agglutination which would certainly be heartily welcomed by medical diagnosticians. Precipitation of albumin from the half-saturated filtrate from the preparation of globulin was effected by the addition of 1.2 cc. of 10 per cent acetic acid per 100 cc. of solution and leaving overnight in the refrigerator. The precipitate was filtered off and redissolved in 100 cc. of distilled water, then reprecipitated by addition of an equal amount of saturated AmSO_4 solution. This precipitate was separated by centrifugalization, redissolved, and dialyzed for 3 hours, but instead of separating upon dialysis it remained in solution. After having obtained this

result in dialyzing, further albumin specimens were used as separated by acetic precipitation, so containing the acetate ion which would render it somewhat more readily soluble than if it were free of electrolyte. However, it appears from the solubility table that the influence of the acetic anions was counteracted by the positive metal ions very close to the isoelectric point, which is given as pH 4.8, and a high degree of dissociation effected in solutions of moderately low pH value.

The precipitates of both proteins were mixed with the solvent and the tubes placed in a 37°C. water bath for 4 hours. Buffer solutions used were Sørensen's

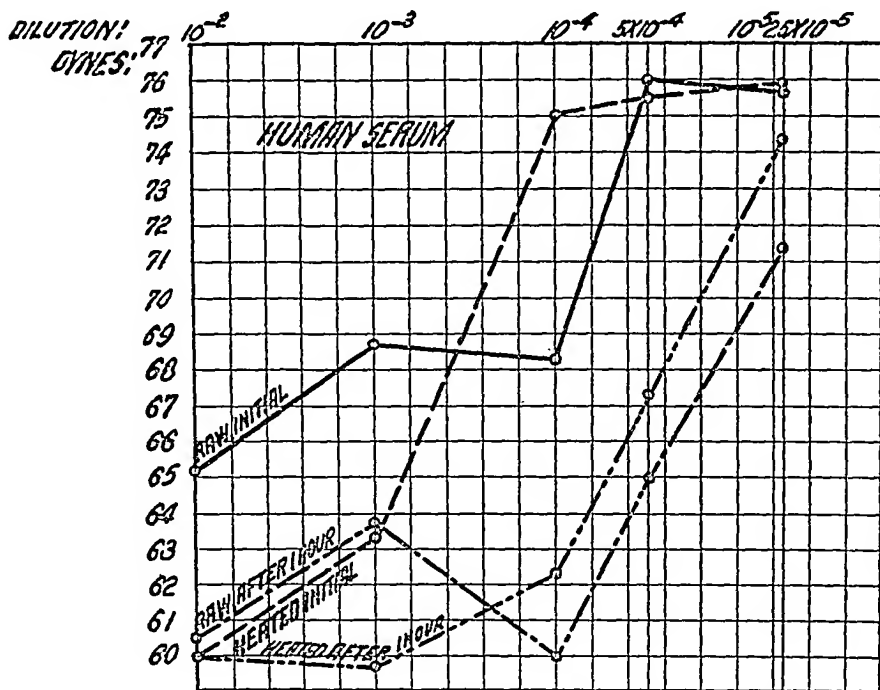


FIG. 7. Effect on surface tension of heating immune serum for $\frac{1}{2}$ hour at 55°C.

citrate and phosphate buffers, and Kolthoff and Vleeschhouwer's soda-borax and alkaline phosphate solutions as given in Clark's text "The determination of hydrogen ions" (16).

Protein determinations were made with Exton's apparatus, the values of which are periodically checked by Kjeldahl's method of nitrogen determination.

This comparison shows definitely that there is a marked difference of dissociability between the two protein fractions of normal serum.

A similar comparison of the solubility of the globulin portion from

SYPHILITIC BLOOD PROTEINS. I

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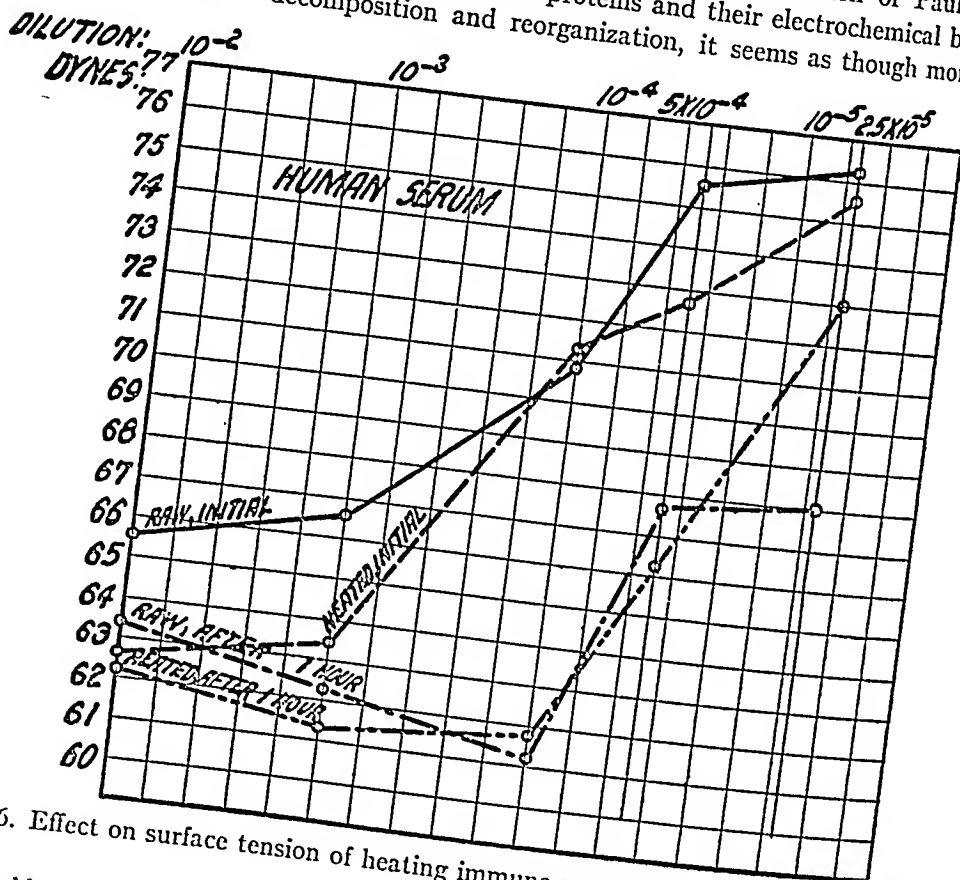


FIG. 6. Effect on surface tension of heating immune serum for $\frac{1}{2}$ hour at 55°C .

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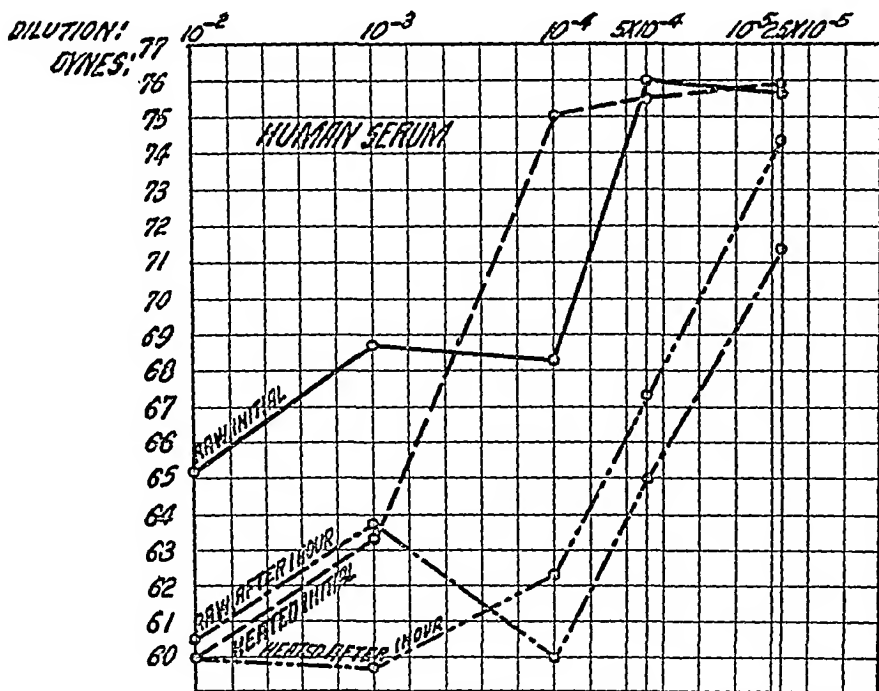


FIG. 7. Effect on surface tension of heating immune serum for $\frac{1}{2}$ hour at 55°C.

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This comparison shows definitely that there is a marked difference of dissociability between the two protein fractions of normal serum.

A similar comparison of the solubility of the globulin portion from

Heating does not affect immune serum in the same manner. The maximum reduction of surface tension in the heated portion occurs at the same dilution or at a lower dilution than for the raw portion. This would tend to indicate that the molecules are not altered in size by immunity. But the fact that separated globulin from syphilitic serum is from 20 to 40 per cent less soluble in buffer solutions of optimum hydrogen ion concentration than that from normal serum in a given time shows that the former is composed of larger aggregates which are more difficult of ionization, and that they may be of different structure.

These results also tend to indicate that the heating of serum before doing a precipitation test brings out a more pronounced flocculation of precipitated particles by causing a greater dissociation of the albumin. The albumin in a more highly ionized state does not interfere with the surface activity as when present in larger aggregates such as occur in the untreated serum.

SUMMARY

These experiments show:

1. That the surface tension of normal blood serum is considerably lowered by standing undisturbed for a period of 1 hour (time-drop).
 2. That the greatest time-drop recorded is with serum diluted approximately 10,000 times in fresh serum, and 50,000 times in heated serum.
 3. That immune serum is not affected in the same manner by heat as is normal serum. Syphilitic serum and anti-sheep cell rabbit serum behave similarly in this respect.
 4. That serum albumin is much more readily soluble in alkaline buffer solutions than globulin is, and that globulin from normal serum ionizes more than that from syphilitic serum.
- Further investigations are being made in an effort to determine why the proteins aggregate or dissociate under the influence of the factors under consideration.

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ORAL IMMUNIZATION AGAINST PNEUMOCOCCUS TYPES II AND III AND THE NORMAL VARIATION IN RESISTANCE TO THESE TYPES AMONG RATS

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A preliminary report on the production of an increased resistance to Types II and III pneumococcus by feeding the organisms to rats has been published elsewhere (1). Maeji, working with Type III, has confirmed this result (2). In the first communication it was stated that rats develop a natural immunity to Type II with increasing age and that this makes it difficult to demonstrate the immunizing effect of feeding the organism. At that time it appeared that a similar natural immunity was not built up against Type III. Since then, it has been found that an analagous resistance to Type III does occur, though it is less extensive and less common than in the case of Type II. This natural, increased tolerance for Types II and III pneumococcus appears at different ages in different rats. It can be observed at weaning time or even sooner toward Type II, and somewhat later in life toward Type III. Several strains of each of these two types, highly virulent for mice, have been used in the experiments reported here.

Variation in Resistance to Types II and III

The resistance to Type II reaches such a height in adult rats of 150 gm. that 10^{-3} cc. or more of a culture highly virulent for mice, may fail to kill. Very often 10^{-8} cc. may kill a very young rat, though it has been observed that equally young rats may not infrequently survive 10^{-5} and 10^{-4} cc. 10^{-8} and 10^{-7} cc. have rarely been observed

* The author wishes to thank Mrs. Lawrance Harriman for kindly providing funds in aid of this work. He is also indebted to the Harriman Research Fund for a grant.

IMMUNIZATION AGAINST PNEUMOCOCCUS II AND III

to kill an adult rat. In the case of Type III, very young rats also generally succumb to 10^{-8} cc., and with increasing age an increased resistance also takes place, but with the strains so far employed adult rats have failed to survive the excessively large quantities that they

TABLE I
Variation in Resistance of Rats to Type II Pneumococcus (Rockefeller Institute Strain)

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
23	10^{-8}	D 4	*41	10^{-8}	S	**17	10^{-8}	D 2
24	10^{-7}	D 4	47	10^{-8}	S	**19	10^{-7}	D 2
24	10^{-6}	D 4	48	10^{-4}	S	**20	10^{-8}	D 2
177	10^{-2}	S	49	10^{-3}	S			
184	10^{-1}	D 2						
197	5x 10^{-1}	D 2	*23	10^{-7}	S	*28	10^{-8}	D 2
*37	10^{-8}	S	22	10^{-8}	S	33	10^{-7}	D 2
39	10^{-7}	S	22	10^{-3}	S	33	10^{-8}	D 3
39	10^{-6}	S	25	10^{-4}	S	36	10^{-8}	D 3
203	10^{-3}	D 3				38	10^{-4}	D 2
229	10^{-2}	D 3	*139	10^{-4}	S	134	10^{-8}	S
231	10^{-1}	D 2	143	10^{-3}	S	134	10^{-7}	S
232	5x 10^{-1}	D 2	144	10^{-2}	S	140	10^{-8}	D 3
			171	10^{-1}	S	165	10^{-5}	D 3
*61	10^{-7}	S	*29	10^{-7}	S	172	10^{-4}	S
72	10^{-6}	S	30	10^{-8}	S	182	10^{-3}	D 2
73	10^{-5}	S	30	10^{-5}	S	180	10^{-2}	S
73	10^{-4}	S	191	10^{-3}	S	182	10^{-1}	D 2
*99	10^{-3}	S	198	10^{-2}	S	221	10^{-8}	S
94	10^{-2}	D 2	199	10^{-1}	S	235	10^{-7}	S
104	10^{-1}	D 2	205	5x 10^{-1}	D 2	235	10^{-6}	D 2
						238	10^{-5}	S
			*44	10^{-8}	S	243	10^{-4}	D 2
			47	10^{-8}	S	252	10^{-3}	D 2
			48	10^{-7}	S	275	10^{-2}	D 2
			48	10^{-7}	S	295	10^{-1}	D 3
			49	10^{-6}	S			
			48	10^{-6}	S			
			50	10^{-5}	S			
			51	10^{-4}	D 2			

S=survived.

* New series.

D=died,—days.

** Mice.

do in the case of Type II. It is often observed, particularly in the case of Type II, that when a number of animals of approximately the same age are injected with increasing doses, there is no sharp demarcation dividing the dose which kills from that which does not, but that

quantities of 10^{-6} and 10^{-5} cc. may kill, whereas the rats receiving 10^{-4} and 10^{-3} cc. may live and that finally, those injected with 10^{-2} and 10^{-1} cc. may succumb.

Table I shows results obtained at different times with the Rockefeller Institute strain of Type II. It can be seen that all the facts noted above are illustrated here, namely (1) some very small rats succumb to 10^{-8} cc., others may survive doses thousands of times as large, (2) large rats are not killed by 10^{-8} and 10^{-7} cc. but generally require thousands of times this quantity, (3) the need for large quan-

TABLE II

Comparative Resistance of Mice, and Young and Adult Rats to Pneumococcus Type II (Rockefeller Institute Strain)

Mice			Young rats			Adult rats		
Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
25	10^{-8}	D 2	50	10^{-8}	S	126	10^{-8}	S
25	10^{-8}	D 2	51	10^{-7}	D 3	118	10^{-8}	S
26	10^{-7}	D 2	57	10^{-6}	S	144	10^{-7}	S
26	10^{-7}	D 2	55	10^{-5}	D 2	152	10^{-7}	S
27	10^{-6}	D 2	61	10^{-4}	D 2	152	10^{-6}	S
27	10^{-6}	D 2	62	10^{-3}	D 3	152	10^{-6}	S
28	10^{-5}	D 2	66	10^{-2}	D 3	157	10^{-5}	S
28	10^{-5}	D 2				161	10^{-5}	S
30	10^{-4}	D 2				157	10^{-4}	S
30	10^{-4}	D 2				165	10^{-4}	D 3*
						171	10^{-3}	S
						169	10^{-3}	S
						197	10^{-2}	S

* Pneumococcus in heart blood.

tities to kill a rat is not owing to a lack of virulence for mice, (4) the irregularity of resistance among rats of approximately the same age (size). Tables II, III, IV, V and VI (using Rockefeller Institute and other strains) further illustrate the facts that, in general the increased resistance appears with increasing age, that the large doses required to kill adult rats are not owing to a diminished virulence for mice in any of the strains used, and that the increased resistance does not appear in rats of approximately the same size at the same time.

The variation in resistance to Type III is less common and appears

later in life than in the case of Type II. Also adult rats have more frequently been found to succumb to smaller doses of the former.

TABLE III
Comparative Resistance of Mice, and Young and Adult Rats to Pneumococcus Type II (Rockefeller Institute Strain)

Mice			Young rats			Adult rats		
Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
26	10 ⁻⁹	D 2	36	10 ⁻⁸	S	109	10 ⁻⁸	S
*28	10 ⁻⁹	D 5	35	10 ⁻⁸	S	115	10 ⁻⁸	S
29	10 ⁻⁸	D 3	38	10 ⁻⁷	D 2	115	10 ⁻⁷	S
29	10 ⁻⁸	D 2	40	10 ⁻⁷	S	118	10 ⁻⁷	S
30	10 ⁻⁸	S	42	10 ⁻⁶	D 3	118	10 ⁻⁶	S
31	10 ⁻⁷	D 2	46	10 ⁻⁶	D 3	119	10 ⁻⁶	S
32	10 ⁻⁷	D 2	52	10 ⁻⁵	S	120	10 ⁻⁵	S
53	10 ⁻⁷	D 2	52	10 ⁻⁵	S	121	10 ⁻⁵	S
33	10 ⁻⁶	D 2	54	10 ⁻⁴	S	121	10 ⁻⁴	S
33	10 ⁻⁶	D 2	58	10 ⁻⁴	D 3	143	10 ⁻⁴	S
33	10 ⁻⁶	D 2	58	10 ⁻³	D 2	**143	10 ⁻³	D 2
34	10 ⁻⁵	D 2	60	10 ⁻²	D 4	146	10 ⁻³	S
34	10 ⁻⁵	D 2				148	10 ⁻²	S
35	10 ⁻⁵	D 2				180	10 ⁻¹	S
36	10 ⁻⁴	D 1						
36	10 ⁻⁴	D 2						

* Contaminant in heart blood.

** Pneumococcus in heart blood.

TABLE IV
Resistance of Mice, and Young and Adult Rats to Pneumococcus Type II (Gill Strain)

Mice			Young rats*			Adult rats		
Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
17	10 ⁻⁹	S	35	10 ⁻⁸	S	142	10 ⁻⁷	S
17	10 ⁻⁹	S	38	10 ⁻⁷	D 2	146	10 ⁻⁶	S
18	10 ⁻⁸	S	38	10 ⁻⁶	D 2	147	10 ⁻⁵	S
18	10 ⁻⁸	D 1	38	10 ⁻⁵	D 1	149	10 ⁻⁴	S
18	10 ⁻⁷	D 2	39	10 ⁻⁴	D 1	152	10 ⁻³	D 2
18	10 ⁻⁷	D 2	42	10 ⁻³	D 1	165	10 ⁻²	S
18	10 ⁻⁶	D 2						
19	10 ⁻⁶	D 2						

* Single litter.

Table VII shows results obtained with the Rockefeller Institute strain of Type III and indicates in general the greater resistance of adult

rats. There is a suggestion of a loss of this immunity later in life, (in the data of the last group) but this may be a matter of chance. The very young rats succumb to the same small doses (10^{-7} and 10^{-8} cc.) which kill mice. The test with the strain Harris (Table VIII)

TABLE V

Resistance of Mice and Young and Adult Rats to Pneumococcus Type II (Weathers Strain)

Mice			Young rats*			Adult rats		
Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
15	10^{-9}	D 2	30	10^{-8}	S	105	10^{-7}	S
16	10^{-9}	S	34	10^{-7}	D 2	111	10^{-8}	S
17	10^{-8}	D 2	34	10^{-8}	D 2	115	10^{-8}	S
18	10^{-8}	S	35	10^{-8}	D 2	119	10^{-8}	D 2
19	10^{-7}	D 2	36	10^{-8}	D 2	128	10^{-8}	S
19	10^{-7}	D 2	36	10^{-8}	D 2	132	10^{-8}	D 2
19	10^{-8}	D 2						
19	10^{-8}	D 2						

* Single litter.

TABLE VI

Resistance of Mice, and Young and Adult Rats to Pneumococcus Type II (Boone Strain)

Mice			Young rats*			Adult rats		
Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
17	10^{-9}	S	34	10^{-8}	D 2	143	10^{-7}	S
17	10^{-9}	S	35	10^{-7}	D 3	143	10^{-8}	S
18	10^{-8}	D 2	36	10^{-8}	D 2	147	10^{-8}	S
19	10^{-8}	D 2	37	10^{-8}	D 2	151	10^{-8}	S
19	10^{-7}	D 2	38	10^{-8}	D 2	154	10^{-8}	S
19	10^{-7}	S	38	10^{-8}	D 2	166	10^{-8}	D 2
19	10^{-8}	D 2						
20	10^{-8}	D 2						

* Single litter.

shows further that adult rats possess an increased tolerance toward Type III, whereas very young ones die of doses no larger than are required to kill mice. The results with the strain Fink (Table IX)

illustrate an instance of a relatively early appearance of the resistance to Type III, 10^{-4} cc. failing to kill a rat weighing only 70 gm.

The experiments with the Challenger and Abaca strains (Table X) illustrate the beginning of the appearance of immunity to Type III

TABLE VII

Resistance of Young and Adult Rats to Pneumococcus Type III (Rockefeller Institute Strain)

Weight	Dose	Result	Weight	Dose	Result
<i>gm.</i>	<i>cc.</i>		<i>gm.</i>	<i>cc.</i>	
27	10^{-8}	S	*32	10^{-8}	D 2
29	10^{-8}	D 2	33	10^{-7}	D 2
29	10^{-7}	D 2	36	10^{-6}	D 2
29	10^{-6}	D 2	36	10^{-5}	D 2
			42	10^{-4}	D 2
138	10^{-3}	S			
157	10^{-4}	S	130	10^{-7}	S
162	10^{-5}	D 6	139	10^{-6}	S
186	10^{-5}	D 2	162	10^{-5}	D 2
			164	10^{-4}	D 2
*36	10^{-8}	D 2	181	10^{-3}	D 2
40	10^{-7}	D 2	184	10^{-2}	S
41	10^{-6}	D 2	187	10^{-2}	D 2
			195	10^{-1}	D 1
108	10^{-7}	S	230	10^{-8}	S
107	10^{-6}	S	285	10^{-7}	D 7
113	10^{-5}	D 3	237	10^{-6}	D 2
			243	10^{-5}	D 2
*38	10^{-8}	D 2	245	10^{-4}	D 2
40	10^{-7}	D 2	262	10^{-3}	S
54	10^{-6}	D 1	285	10^{-2}	D 2
			305	10^{-1}	D 1
126	10^{-7}	S			
129	10^{-6}	S			
131	10^{-5}	D 2			
*51	10^{-8}	S			
55	10^{-7}	D 2			
58	10^{-6}	D 2			
122	10^{-7}	S			
125	10^{-6}	S			
129	10^{-5}	S			

* New series.

with increasing age, in the former by the survival of one of each of 2 rats injected with 10^{-7} and 10^{-6} cc. respectively, and in the latter by the survival of the rats injected with 10^{-8} and 10^{-7} cc. It can be seen that the virulence for mice is high. The strain Small (Table XI)

seemed at first to be equally toxic for mice, and young and adult rats, killing all 3 in a dose of 10^{-7} cc. (upper half of table). Another group of animals was tested and it was found that although 10^{-8} cc. killed both young rats, 1 survived 10^{-7} cc. and 1 adult rat failed to die of

TABLE VIII

Resistance of Mice and Young and Adult Rats to Pneumococcus Type III (Harris Strain)

Mice			Young rats*			Adult rats		
Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
16	10^{-8}	S	29	10^{-8}	D 2	132	10^{-8}	S
17	10^{-8}	S	30	10^{-8}	S	135	10^{-7}	S
17	10^{-8}	S	32	10^{-7}	D 2	138	10^{-6}	S
19	10^{-8}	D 2	32	10^{-6}	D 2	138	10^{-5}	S
19	10^{-7}	D 2	34	10^{-5}	D 2	145	10^{-4}	D 2
19	10^{-6}	D 2	35	10^{-4}	D 2	148	10^{-4}	D 2
20	10^{-6}	D 2						

* A single litter.

TABLE IX

Resistance of Mice, and Young and Adult Rats to Pneumococcus Type III (Fink Strain)

Mice			Young rats*			Adult rats		
Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
17	10^{-8}	S	59	10^{-9}	S	143	10^{-8}	S
20	10^{-8}	S	66	10^{-8}	S	141	10^{-7}	S
22	10^{-8}	S	60	10^{-8}	S	157	10^{-6}	S
21	10^{-8}	S	67	10^{-7}	S	165	10^{-5}	S
24	10^{-7}	D 2	68	10^{-6}	S	170	10^{-4}	D 4
23	10^{-7}	D 2	68	10^{-5}	S			
25	10^{-6}	D 2	70	10^{-4}	S			

* A single litter.

10^{-6} cc., showing that rats may develop the characteristic immunity towards this strain as well. This is substantiated by the data in Table XXIII obtained in a feeding experiment where several controls survived 10^{-5} and 10^{-6} cc., although others died of 10^{-8} and 10^{-7} cc.

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TABLE X
Resistance of Mice and Rats to *Pneumococcus* Type III (Challenger and Strains)

Challenger											
Mice			Rats			Abaca					
Weight	Dose	Result	Weight	Dose	Result	Mice			Rats		
gm.	cc.		gm.	cc.		gm.	cc.		gm.	cc.	Res
14	10 ⁻⁸	S	79	10 ⁻⁸	D 3	15	10 ⁻⁸	D 3	68	10 ⁻⁸	S
14	10 ⁻⁸	D 2	90	10 ⁻⁷	S	16	10 ⁻⁸	D 3	77	10 ⁻⁷	S
13	10 ⁻⁸	S	91	10 ⁻⁷	D 2	16	10 ⁻⁸	D 2	80	10 ⁻⁷	S
14	10 ⁻⁷	D 2	97	10 ⁻⁸	D 2	16	10 ⁻⁷	D 2	95	10 ⁻⁸	D 2
14	10 ⁻⁷	D 2	94	10 ⁻⁸	S	16	10 ⁻⁷	D 2	93	10 ⁻⁸	D 2
15	10 ⁻⁸	D 2	101	10 ⁻⁸	D 2	17	10 ⁻⁷	D 2	110	10 ⁻⁸	D 2
15	10 ⁻⁸	D 2	111	10 ⁻⁵	D 3	17	10 ⁻⁷	D 2	113	10 ⁻⁸	D 2
15	10 ⁻⁸	D 2	117	10 ⁻⁴	D 2	17	10 ⁻⁸	D 2	115	10 ⁻⁸	D 2
14	10 ⁻⁸	D 2	116	10 ⁻⁴	D 2	17	10 ⁻⁸	D 2	142	10 ⁻⁴	D 2
15	10 ⁻⁸	D 2	133	10 ⁻³	D 2	18	10 ⁻⁸	D 2	191	10 ⁻³	D 2
15	10 ⁻⁸	D 2	124	10 ⁻³	D 2	18	10 ⁻⁸	D 1	150	10 ⁻³	D 2
15	10 ⁻⁸	D 1				18	10 ⁻⁸	D 1			

TABLE XI
Resistance of Mice, and Young and Adult Rats to *Pneumococcus* Type III (Small Strain)

Mice			Young rats*					
Weight	Dose	Result	Weight	Dose	Result	Adult rats		
gm.	cc.		gm.	cc.		Weight	Dose	Result
**16	10 ⁻⁸	S	34	10 ⁻⁸	S	132	cc.	
18	10 ⁻⁸	S	37	10 ⁻⁷	D 2	133	10 ⁻⁸	S
20	10 ⁻⁷	D 2	51	10 ⁻⁸	D 1	149	10 ⁻⁷	D 2
21	10 ⁻⁸	D 1	53	10 ⁻⁸	D 1	157	10 ⁻⁸	D 2
21	10 ⁻⁸	D 2				160	10 ⁻⁸	D 1
**13							10 ⁻⁴	D 2
16	10 ⁻⁸	S	32	10 ⁻⁸	D 2	127	10 ⁻⁷	D 2
16	10 ⁻⁸	S	32	10 ⁻⁸	D 2	128	10 ⁻⁸	S
17	10 ⁻⁷	D 3	35	10 ⁻⁷	D 3	136	10 ⁻⁸	D 1
19	10 ⁻⁷	D 3	37	10 ⁻⁷	S	140	10 ⁻⁴	D 2
22	10 ⁻⁸	D 3	39	10 ⁻⁸	D 2	155	10 ⁻⁸	D 1
			39	10 ⁻⁸	D 1			

* A single litter.

** Two tests done on different days.

Immunity to *Pneumococcus* Types II and III after Ingestion of the Acid-Killed or of Bile Salt-Dissolved Organisms

The high degree of natural immunity of all but very young rats to Types II and III, particularly to the former, makes it more difficult to

demonstrate clearly the increased resistance which follows feeding of these types than when Type I is employed. If one takes a group of rats at random for example, one may find that the control and treated rats survive equally large doses especially in the case of Type II. This is so because a change from a resistance equal to 1000 M.L.D. to that of 2000 M.L.D., which may have been the effect of feeding the bacteria, is not detectable by the method employed. Even if one chooses very young rats of different litters and mixes them, this difficulty is avoided only fairly well in the case of Type III, and not in the case of Type II.

By using single young litters and dividing into controls and experimental animals it has been possible to show that the ingestion of Type II pneumococci is followed by a definitely increased resistance to this type. This precaution is not necessary in the case of Type III, though desirable, and by using young rats (60 gm.) of different mothers, mixing them and dividing into controls and experimentals, an analogous result is obtained.

When the first experiments were begun with Types II and III, 15 feedings were employed. At the end of this time part of the natural immunity would appear even if young animals were used. After it was found that a single feeding was sufficient for producing an immunity against Type I, the same or a slightly larger number were tried for Types II and III. This removed another interfering factor.

In all the experiments described here the cultures were killed by adding N HCl to make N/12 and leaving at room temperature for 2 hours, and then centrifuging. The media used were either beef heart broth or 0.3 per cent glucose meat extract broth. Wherever bile salt-dissolved organisms were employed they were grown only in beef heart broth. The cultures for injection were grown in beef heart broth enriched with blood, and injections were made intraperitoneally in a volume of 0.20 cc., dilutions being made in beef heart broth.

Experiments with Type II

Experiment 1, Table XII.—Each of 3 litters of 8 was divided into 4 controls and 4 treated rats. The organism was grown in glucose meat extract broth and the bacteria were mixed with cracker meal. Controls were fed the cracker meal alone. The deaths are very regular among the controls, with the exception of 10^{-6} cc. in 1st litter. There were 6 deaths among the untreated and only 1 among the treated rats.

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Experiment 2, Table XIII.—Each of 2 litters was divided into controls and treated rats. Organisms were grown in beef heart broth, and mixed with cracker meal. In the 1st litter controls were killed by 10^{-8} , 10^{-7} , 10^{-6} and 10^{-5} cc.

TABLE XII
Resistance to Pneumococcus Type II Following Ingestion of the Acid-Killed Organisms

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C28	10^{-8}	S	C49	10^{-8}	S	C32	10^{-8}	S
C37	10^{-7}	D 2	C53	10^{-7}	*	C33	10^{-7}	S
C42	10^{-6}	S	C55	10^{-6}	D 2	C36	10^{-6}	D 2
C43	10^{-5}	D 2	C65	10^{-5}	D 2	C37	10^{-5}	D 1
E37	10^{-8}	S	E48	10^{-8}	S	E30	10^{-8}	S
E38	10^{-7}	S	E53	10^{-7}	S	E32	10^{-7}	S
E42	10^{-6}	S	E56	10^{-6}	S	E32	10^{-6}	S
E43	10^{-5}	D 2	E60	10^{-5}	S	E34	10^{-5}	S

C = control. E = treated rat. S = survived. D = died,—days.
Each group of C and E rats is a litter. Each E rat received bacteria from 5 cc. growth on each of 2 successive days. Test was done 3 days after 2nd feeding.
* Missing, probably died.

TABLE XIII
Resistance to Pneumococcus Type II Following Ingestion of the Acid-Killed Organisms

Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.	
C38	10^{-8}	D 2	C34	10^{-8}	S
C40	10^{-7}	D 2	C35	10^{-7}	S
C40	10^{-6}	D 2	C36	10^{-6}	D 2
C49	10^{-5}	D 2	C41	10^{-5}	D 2
E38	10^{-8}	S	E35	10^{-7}	S
E38	10^{-7}	S	E40	10^{-6}	S
E39	10^{-6}	S	E41	10^{-5}	S
E42	10^{-5}	S			

Each group of C and E rats is a litter. Each E rat received bacteria from 5 cc. growth on each of 3 successive days. Test was done 48 hours after 3rd dose.

Treated animals survived these quantities. In the 2nd litter 10^{-7} and 10^{-6} cc. proved fatal for controls but not for treated animals.

Experiment 3, Table XIV.—Each of 3 litters was divided into 4 controls and 4 treated rats. The organisms were grown in beef heart broth and were suspended

in milk. The suspension was administered by medicine dropper. Controls died regularly of 10^{-7} , 10^{-6} and 10^{-5} cc., with one exception in the 3rd litter. The treated animals survived 10^{-7} and 10^{-6} cc. in all 3 litters but succumbed to 10^{-5} cc. There were 8 deaths among controls as against 3 among the treated rats.

TABLE XIV

Resistance to Pneumococcus Type II Following Ingestion of the Acid-Killed Organisms

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C39	10^{-8}	S	C36	10^{-8}	S	C30	10^{-8}	S
C41	10^{-7}	D 2	C38	10^{-7}	D 1	C32	10^{-7}	D 1
C44	10^{-6}	D 2	C42	10^{-6}	D 2	C33	10^{-6}	S
C50	10^{-5}	D 1	C45	10^{-5}	D 1	C34	10^{-5}	D 2
E42	10^{-8}	S	E37	10^{-8}	S	E28	10^{-8}	S
E43	10^{-7}	S	E38	10^{-7}	S	E30	10^{-7}	S
E43	10^{-6}	S	E39	10^{-6}	S	E31	10^{-6}	S
E43	10^{-5}	D 2	E40	10^{-5}	D 2	E31	10^{-5}	D 2

Each group of C and E rats is a litter. Each E rat received bacteria from 5 cc. growth on each of 3 successive days. Test was done 1 day after last feeding.

TABLE XV

Resistance to Pneumococcus Type II Following Ingestion of the Acid-Killed Organisms

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C37	10^{-8}	S	C34	10^{-8}	D 2	C27	10^{-8}	D 1
C41	10^{-7}	D 2	C31	10^{-7}	S	C29	10^{-7}	D 2
C45	10^{-6}	D 1	C34	10^{-6}	D 1	C32	10^{-6}	D 1
C46	10^{-5}	D 3	C35	10^{-5}	S	C33	10^{-5}	D 1
E38	10^{-8}	S	E31	10^{-8}	D 2	E26	10^{-8}	D 1
E39	10^{-7}	S	E33	10^{-7}	S	E28	10^{-7}	D 2
E42	10^{-6}	S	E33	10^{-6}	S	E31	10^{-6}	S
E48	10^{-5}	S	E34	10^{-5}	S	E34	10^{-5}	S

Each group of C and E rats is a litter. Each E rat received the bacteria from 5 cc. growth on each of 3 successive days. Test was done 48 hours after 3rd feeding.

Experiment 4, Table XV.—Each of 3 litters was divided into 4 controls and 4 treated rats. The organisms were grown in beef heart broth and were mixed with cracker meal. There were 9 deaths among the controls and only 3 among the treated rats.

Three experiments were done in which the Berkefeld V filtrate of sodium glycocholate-dissolved organisms was fed. For Type I such filtrates were found to immunize as well as the entire cell (3). Powdered sodium glycocholate was added to the centrifuged living Type II organisms and after solution was complete water was added, the whole allowed to stand for a while and then filtered. Cracker meal was added to this until the mass was of pasty consistency, and each rat as usual was fed in an individual cage.

TABLE XVI
Resistance to Pneumococcus Type II, Following Ingestion of Berkefeld Filtrate of the Sodium Glycocholate-Dissolved Organisms

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm	cc.		gm.	cc.		gm.	cc.	
C32	10 ⁻⁸	S	C36	10 ⁻⁸	S	C37	10 ⁻⁸	D 2
C34	10 ⁻⁷	D 2	C40	10 ⁻⁷	D 3	C39	10 ⁻⁷	D 2
C37	10 ⁻⁶	D 2	C41	10 ⁻⁶	S	C40	10 ⁻⁶	D 2
C40	10 ⁻⁵	D 2	C42	10 ⁻⁵	D 2	C41	10 ⁻⁵	D 2
E27	10 ⁻⁸	S	E36	10 ⁻⁸	S	E40	10 ⁻⁸	S
E33	10 ⁻⁷	S	E38	10 ⁻⁷	S	E39	10 ⁻⁷	S
E34	10 ⁻⁶	D 2	E42	10 ⁻⁶	D 2	E40	10 ⁻⁶	S
E38	10 ⁻⁵	D 2	E42	10 ⁻⁵	S	E43	10 ⁻⁵	S

Each group of C and E rats is a litter. Each E rat received the equivalent of 10 cc. growth on each of 3 successive days. Test was done 1 day after last feeding.

Experiment 5, Table XVI.—Each of 3 litters was divided into control and treated rats. Deaths were regular among controls with the exception of 10⁻⁶ cc. rat in the 2nd litter. There were 9 deaths among the controls and 3 among the treated animals.

Experiment 6, Table XVII.—Each of 2 litters was divided into controls and treated rats. In each litter 10⁻⁶ and 10⁻⁵ cc. were fatal for controls while all the treated rats lived. 2 deaths in which a contaminating organism was found at autopsy are not counted (1 C and 1 E).

Experiment 7, Table XVIII.—Each of 3 litters was divided into controls and experimental animals. There were 7 deaths among the former and 1 among the latter.

It is evident from these experiments that rats develop an increased resistance to *Pneumococcus* Type II, following the ingestion of either the dead whole organisms or the Berkefeld filtrate of the bile salt-dissolved bacteria.

In order to learn whether cross-protection existed between Types I and II, three experiments were done, one in which Type II was fed and two in which Type I was administered.

TABLE XVII

Resistance to Pneumococcus Type II, Following Ingestion of Berkefeld Filtrate of Sodium Glycocholate-Dissolved Organisms

Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.	
C31	10 ⁻⁸	S	**C36	10 ⁻⁸	D 3
C32	10 ⁻⁷	S	C39	10 ⁻⁷	S
*C39	10 ⁻⁸	D 1	*C44	10 ⁻⁸	D 2
C40	10 ⁻⁸	D 2	C47	10 ⁻⁸	D 1
E28	10 ⁻⁸	S	E37	10 ⁻⁸	S
E29	10 ⁻⁷	S	E41	10 ⁻⁷	S
**E35	10 ⁻⁸	D 4	E42	10 ⁻⁸	S
E35	10 ⁻⁸	S	E46	10 ⁻⁸	S

Each group of C and E rats is a litter. Each E rat received the equivalent of 10 cc. growth on each of 3 successive days. Test was done 2 days after last feeding.

* Pneumococcus in heart blood.

** Contaminant in heart blood. Both of these rats were ill before the injection.

TABLE XVIII

Resistance to Pneumococcus Type II, Following Ingestion of Berkefeld Filtrate of Sodium Glycocholate-Dissolved Organisms

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C37	10 ⁻⁸	S	C33	10 ⁻⁸	S	C41	10 ⁻⁸	S
C40	10 ⁻⁷	D 1	C34	10 ⁻⁷	D 2	C43	10 ⁻⁷	D 2
C44	10 ⁻⁸	D 1	C43	10 ⁻⁸	S	C55	10 ⁻⁸	D 3
C44	10 ⁻⁸	D 2	C43	10 ⁻⁸	D 1	C57	10 ⁻⁸	S
E39	10 ⁻⁸	S	E33	10 ⁻⁸	S	E31	10 ⁻⁸	S
E42	10 ⁻⁷	S	E34	10 ⁻⁷	D 2	E42	10 ⁻⁷	S
E45	10 ⁻⁸	S	E35	10 ⁻⁸	S	E49	10 ⁻⁸	S
E46	10 ⁻⁸	S	E37	10 ⁻⁸	S	E54	10 ⁻⁸	S

Each group of C and E rats is a litter. Each E rat received the equivalent of 10 cc. growth on each of 2 successive days. Test was done 2 days after the 2nd feeding.

Experiment 8, Table XIX.—Large quantities (see table) of HCl-killed Type II organisms grown in glucose meat extract broth were fed to rats but it is obvious that little or no increased resistance to Type I resulted.

Experiment 9, Table XX.—Each of 3 litters was divided into controls and

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TABLE XIX
Resistance to *Pneumococcus* Type I, Following Ingestion of *Pneumococcus* Type II

Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.	
C117	10 ⁻⁸	D 2	*C142	10 ⁻⁸	D 2
C125	10 ⁻⁷	D 2	C143	10 ⁻⁷	D 2
E130	10 ⁻⁶	D 2	C144	10 ⁻⁶	D 2
E151	10 ⁻⁶	D 2	C158	10 ⁻⁵	D 2
*C121	10 ⁻⁸	S	E142	10 ⁻⁵	D 2
C127	10 ⁻⁷	S	E156	10 ⁻⁷	D 2
C130	10 ⁻⁶	D 2	E143	10 ⁻⁶	D 2
E112	10 ⁻⁷	D 2	E165	10 ⁻⁶	D 2
E131	10 ⁻⁶	D 2		10 ⁻⁵	D 2

Each treated rat was fed the equivalent of 50 cc. acid-killed growth per day. In the first group the 2 E rats received 16 such feedings over a period of 21 days. The 2 E rats in the second group received 17 feedings over a period of 22 days and the 4 E rats in the last group received 19 feedings over a period of 25 days. In each group the test was done on the day following the last feeding.

* New series.

TABLE XX
Resistance to *Pneumococcus* Type II, Following Ingestion of *Pneumococcus* Type I

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C32	10 ⁻⁸	S	C26	10 ⁻⁸	S	C30	10 ⁻⁸	S
C34	10 ⁻⁷	D 2	C34	10 ⁻⁷	D 2	C31	10 ⁻⁷	S
C35	10 ⁻⁶	D 2	C34	10 ⁻⁶	D 2	C35	10 ⁻⁶	D 2
C35	10 ⁻⁵	S	C37	10 ⁻⁵	D 2	C41	10 ⁻⁵	D 2
E31	10 ⁻⁸	D 2	E34	10 ⁻⁸	S	E29	10 ⁻⁸	S
E36	10 ⁻⁷	D 1	E36	10 ⁻⁷	*	E31	10 ⁻⁷	D 2
E37	10 ⁻⁶	D 1	E38	10 ⁻⁶	D 4	E37	10 ⁻⁶	S
E38	10 ⁻⁵	S	E42	10 ⁻⁵	D 1	E38	10 ⁻⁵	D 1

Each group of C and E rats is a litter. Each E rat was fed the bacteria from 10 cc. growth on each of 2 successive days. Test was done 2 days after 2nd feeding.

* Missing, probably dead.

treated animals. Type I organisms grown in beef heart broth and killed by contact with N/12 HCl were fed. There was a total of 7 deaths among controls and 7 among the treated animals.

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Experiment 10, Table XXI.—Each of 3 litters was divided into control and treated animals. Type I organisms, grown in glucose meat extract broth, mixed with N/12 HCl, centrifuged and mixed with cracker meal were fed. There were no deaths among the controls and 9 among the treated rats.

TABLE XXI
Resistance to Pneumococcus Type II, Following Ingestion of Pneumococcus Type I

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C36	10 ⁻⁸	S	C36	10 ⁻⁸	D 2	C28	10 ⁻⁸	S
C38	10 ⁻⁷	S	C37	10 ⁻⁷	S	C37	10 ⁻⁷	S
C40	10 ⁻⁶	D 1	C42	10 ⁻⁶	D 1	C40	10 ⁻⁶	S
C42	10 ⁻⁵	D 1	C47	10 ⁻⁶	D 1	C47	10 ⁻⁶	S
E37	10 ⁻⁸	D 2	E36	10 ⁻⁸	D 2	E33	10 ⁻⁸	S
E40	10 ⁻⁷	D 2	E39	10 ⁻⁷	S	E35	10 ⁻⁷	S
E46	10 ⁻⁶	D 2	E41	10 ⁻⁶	D 3	E39	10 ⁻⁶	S
E50	10 ⁻⁵	D 1	E46	10 ⁻⁵	D 1	E44	10 ⁻⁵	S

Each group of C and E rats in a litter. Each E rat was fed the bacteria from 10⁻⁸ to 10⁻⁵ cc. growth on each of 3 successive days. Test was done 2 days after the last feeding.

It is plain that no evidence for any increased resistance against Type II following ingestion of Type I was obtained.

Experiments with Type III

Experiment 1, Table XXII.—22 rats of approximately the same age were divided equally into controls and treated groups. Organisms were grown in beef broth, centrifuged and mixed with cracker meal. Of the 11 controls only 3 died, 1 each with 10⁻⁸, 10⁻⁷ and 10⁻⁶ cc. Of the 11 treated rats only 1 died.

Experiment 2, Table XXIII.—22 rats of approximately the same age were divided equally into controls and treated animals. In order to learn whether desiccation of the bacteria and feeding together with other types would interfere with the immunizing effect, Types I, II, III, IV and V were grown in glucose meat extract broth and administered as a powder. The powder was suspended in water and mixed with cracker meal. In spite of the irregularity in resistance to Type III among control rats it seems clear that the treated animals, with one exception, died or death among the 11 injected, were immunized. The results seem to show a noticeable loss of immunizing value owing to desiccation, or simultaneous feeding with other types.¹

¹ A similar experiment in which resistance against Type I was tested showed a loss of immunizing action.

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TABLE XXII

Resistance to Pneumococcus Type III, Following Ingestion of the Acid-Killed Organisms

Weight	Dose	Result	Weight	Dose	Result
<i>gm.</i>	<i>cc.</i>		<i>gm.</i>	<i>cc.</i>	
C78	10 ⁻⁸	D 2	E77	10 ⁻⁸	S
C80	10 ⁻⁸	S	E86	10 ⁻⁸	S
C88	10 ⁻⁷	D 2	E88	10 ⁻⁷	S
C87	10 ⁻⁷	S	E92	10 ⁻⁷	S
C95	10 ⁻⁶	D 2	E94	10 ⁻⁷	S
C90	10 ⁻⁶	S	E95	10 ⁻⁷	S
C98	10 ⁻⁶	D 2	E95	10 ⁻⁶	S
C107	10 ⁻⁶	D 2	E95	10 ⁻⁶	S
C101	10 ⁻⁵	D 1	E98	10 ⁻⁵	S
C101	10 ⁻⁵	D 2	E119	10 ⁻⁵	D 1
C110	10 ⁻⁵	D 2	E111	10 ⁻⁵	S

Each E rat received the organisms from 10 cc. growth on each of 3 successive days.
 Test was done 1 day after last feeding.

TABLE XXIII

*Resistance to Pneumococcus Type III, Following Ingestion of the Acid-Killed Organisms of Types I, II, III, IV and V**

Weight	Dose	Result	Weight	Dose	Result
<i>gm.</i>	<i>cc.</i>		<i>gm.</i>	<i>cc.</i>	
C92	10 ⁻⁸	D 2	E90	10 ⁻⁸	S
C94	10 ⁻⁸	S	E85	10 ⁻⁸	S
C94	10 ⁻⁷	D 2	E95	10 ⁻⁷	S
C100	10 ⁻⁷	D 2	E101	10 ⁻⁷	S
C101	10 ⁻⁷	D 2	E99	10 ⁻⁷	S
C108	10 ⁻⁶	D 2	E108	10 ⁻⁷	S
C107	10 ⁻⁶	D 2	E110	10 ⁻⁶	S
C111	10 ⁻⁶	S	E107	10 ⁻⁶	S
C115	10 ⁻⁵	S	E121	10 ⁻⁶	S
C125	10 ⁻⁵	D 2	E114	10 ⁻⁵	S
C113	10 ⁻⁵	S	E115	10 ⁻⁵	D 2
				10 ⁻⁵	S

A desiccated powder was fed. This contained HCl-killed *Pneumococcus* Types I, II, III, IV and V. Each E rat received the bacteria from 5 cc. growth of each type on each of 3 successive days. Test was done 2 days after last feeding.

* Type numbers are those used at Bureau of Laboratories, Department of Health. See Cooper, G., Edwards, M., and Rosenstein, C., *J. Exp. Med.*, 1929, 49, 461.

Experiment 3, Table XXIV.—The details of this experiment are like those in the preceding one. There is less irregularity in resistance among the controls than in Experiment 2. There were 7 survivors among the treated animals and 4 among the controls.

Experiment 4, Table XXV.—The details are the same as for Experiment 1, except for the quantity of bacteria fed and the time of the test. With a single

TABLE XXIV

Resistance to Pneumococcus Type III, Following Ingestion of the Acid-Killed Organisms of Types I, II, III, IV and V

Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.	
C72	10^{-8}	S	E75	10^{-8}	S
C77	10^{-8}	D 2	E75	10^{-8}	S
C78	10^{-7}	S	E78	10^{-7}	S
C79	10^{-7}	D 2	E79	10^{-7}	S
C79	10^{-7}	S	E80	10^{-7}	S
C82	10^{-8}	S	E82	10^{-8}	S
C85	10^{-8}	D 2	E81	10^{-8}	S
C84	10^{-8}	D 2	E82	10^{-8}	D 2
C86	10^{-8}	D 2	E86	10^{-8}	D 2
C89	10^{-8}	D 2	E89	10^{-8}	D 2
C85	10^{-8}	D 2	E90	10^{-8}	D 2

A desiccated powder was fed. This contained the acid-killed organisms of Types I, II, III, IV and V. Each E rat was fed the bacteria from 5 cc. growth of each type on each of 3 successive days. Test was done 3 days after last feeding.

TABLE XXV

Resistance to Pneumococcus Type III, Following Ingestion of the Acid-Killed Organism

Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.	
C83	10^{-8}	D 2	E80	10^{-8}	S
C81	10^{-8}	S	E85	10^{-8}	S
C90	10^{-7}	D 2	E85	10^{-7}	S
C85	10^{-7}	D 2	E88	10^{-7}	S
C88	10^{-7}	D 2	E95	10^{-7}	S
C90	10^{-8}	S	E95	10^{-8}	S
C90	10^{-8}	D 2	E93	10^{-8}	S
C90	10^{-8}	D 2	E100	10^{-8}	D 2
C93	10^{-8}	D 2	E100	10^{-8}	S
C103	10^{-8}	D 2	E103	10^{-8}	D 2
C98	10^{-8}	D 2	E102	10^{-8}	S
			E101	10^{-8}	S

Each E rat received the bacteria from 5 cc. growth on each of 3 successive days. Test was done 3 days after last feeding.

exception (10^{-6} cc.) the controls died uniformly in this group. There were 2 survivors among the 11 controls and 10 among the 12 immunized animals.

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Experiment 5, Table XXVI.—There were 3 litters in this experiment, each being divided equally into controls and treated animals. The organisms were grown in beef heart broth, centrifuged, suspended in milk and fed by medicine dropper. Deaths among the controls are quite regular, all but 1 of 12 dying (10^{-8} cc.). Among the treated rats 3 out of 12 died.

TABLE XXVI
Resistance to Pneumococcus Type III, Following Ingestion of the Acid-Killed Organisms

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C63	10^{-8}	D 2	C73	10^{-8}	D 2	C49	10^{-8}	S
C71	10^{-7}	D 1	C80	10^{-7}	D 2	C50	10^{-7}	D 2
C77	10^{-6}	D 1	C82	10^{-6}	D 2	C61	10^{-6}	D 1
C79	10^{-5}	D 1	C92	10^{-5}	D 1	C68	10^{-5}	D 1
E51	10^{-8}	S	E75	10^{-8}	S	E50	10^{-8}	S
E61	10^{-7}	S	E77	10^{-7}	S	E51	10^{-7}	S
E75	10^{-6}	S	E78	10^{-6}	D 1	E54	10^{-6}	S
E77	10^{-5}	D 1	E83	10^{-5}	D 1	E60	10^{-5}	S

Each E rat received the bacteria from 5 cc. growth on each of 3 successive days. Test was done 1 day after last feeding. Each group of C and E rats is a litter.

These data substantiate the earlier results and show that an immunity can be obtained in rats by oral administration of dead Type III pneumococci. Additional experiments were done with the Berkefeld filtrate (V) of sodium glycocholate-dissolved bacteria. The manner of preparing this filtrate was the same as described above for Type II.

TABLE XXVII
Resistance to Pneumococcus Type III, Following Ingestion of Berkefeld Filtrate of the Sodium Glycocholate-Dissolved Organisms

Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.	
C39	10^{-8}	S	E44	10^{-8}	S
C40	10^{-7}	D 3	E46	10^{-7}	S
C42	10^{-6}	D 2	E50	10^{-6}	S
C46	10^{-5}	D 2	E51	10^{-5}	D 1
C52	10^{-4}	D 2	E53	10^{-4}	D 1
			E50	10^{-4}	D 2

Each E rat received the equivalent of 20 cc. growth 48 hours before the test.

Experiment 6, Table XXVII.—6 young rats were given a single feeding of the filtrate mixed with milk and containing 2 mg. of sodium glycocholate. The controls were fed sodium glycocholate alone in equal amounts in a similar manner. 1 control and 3 treated rats survived.

TABLE XXVIII

Resistance to Pneumococcus Type III, Following Ingestion of the Berkefeld Filtrate of the Sodium Glycocholate-Dissolved Organisms

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C33	10 ⁻⁸	S	C39	10 ⁻⁸	S	C55	10 ⁻⁸	D 2
C33	10 ⁻⁷	S	C41	10 ⁻⁷	D 2	C61	10 ⁻⁷	S
C34	10 ⁻⁶	D 2	C45	10 ⁻⁶	D 2	C70	10 ⁻⁶	D 3
C37	10 ⁻⁵	D 5	C49	10 ⁻⁵	S	C74	10 ⁻⁵	D 2
C38	10 ⁻⁵	D 2	C53	10 ⁻⁵	D 2	C85	10 ⁻⁵	S
E30	10 ⁻⁸	S	E45	10 ⁻⁷	S	E64	10 ⁻⁷	S
E35	10 ⁻⁷	S	E45	10 ⁻⁶	S	E65	10 ⁻⁷	S
E36	10 ⁻⁶	S	E54	10 ⁻⁶	D 2	E71	10 ⁻⁶	S
E41	10 ⁻⁵	S	E60	10 ⁻⁵	S	E79	10 ⁻⁵	S
E36	10 ⁻⁵	D 2	E62	10 ⁻⁵	S	E83	10 ⁻⁵	D 3

The test was done 4 days after a single feeding, equivalent to 20 cc. growth, per rat.

TABLE XXIX

Resistance to Pneumococcus Type III, Following Ingestion of the Berkefeld Filtrate of the Sodium Glycocholate-Dissolved Organisms

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C10	10 ⁻⁸	D 3	C24	10 ⁻⁸	D 1	C17	10 ⁻⁸	S
C12	10 ⁻⁷	D 2	C24	10 ⁻⁷	D 1	C20	10 ⁻⁸	D 1
E11	10 ⁻⁸	S	E20	10 ⁻⁸	S	C20	10 ⁻⁷	D 1
E13	10 ⁻⁷	S	E26	10 ⁻⁷	S	C21	10 ⁻⁶	D 1
E12	10 ⁻⁷	D 2	E27	10 ⁻⁶	D 1	E22	10 ⁻⁸	S
						E23	10 ⁻⁷	S
						E23	10 ⁻⁶	S
						E24	10 ⁻⁵	D 1

A single feeding equivalent to 5 cc. growth was given by medicine dropper 48 hours previously. Each group of C and E rats is a litter.

Experiment 7, Table XXVIII.—A group of rats were divided into 3 parts and each of these was subdivided into controls and treated animals of approximately the same range of weights. The Type III pneumococcus was grown in beef heart broth, centrifuged and dissolved in sodium glycocholate (0.75 mg. for 20 cc.).

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Deaths among controls were fairly regular and 9 out of 15 succumbed as against 3 out of 15 among the treated animals.

Experiment 8, Table XXIX.—Each of 3 litters was divided into controls and treated animals. The pneumococcus was grown in beef heart broth, and dissolved in sodium glycocholate (0.5 mg. for organisms from 5 cc.). The animals were fed the Berkefeld filtrate mixed with milk, by medicine dropper. 7 out of 8 controls and 3 out of 10 treated rats died.

Experiment 9, Table XXX.—Each of 3 litters was divided into control and experimental animals. The Type III pneumococcus was grown in beef heart broth, dissolved in sodium glycocholate (1 mg. per day per rat) and mixed with cracker meal. There was only 1 death among the treated rats as against 7 among the controls.

TABLE XXX
Resistance to Pneumococcus Type III, Following Ingestion of Berkefeld Filtrate of the Sodium Glycocholate-Dissolved Organisms

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C35	10 ⁻⁸	S	C36	10 ⁻⁸	D 2	C31	10 ⁻⁸	S
C38	10 ⁻⁷	S	C38	10 ⁻⁷	D 2	C33	10 ⁻⁷	D 2
C38	10 ⁻⁶	D 2	C42	10 ⁻⁶	S	C36	10 ⁻⁶	S
C42	10 ⁻⁵	D 1	C45	10 ⁻⁵	D 2	C38	10 ⁻⁵	D 2
E35	10 ⁻⁸	S	E36	10 ⁻⁸	S	E26	10 ⁻⁸	S
E36	10 ⁻⁷	S	E41	10 ⁻⁷	S	E29	10 ⁻⁷	S
E38	10 ⁻⁶	S	E43	10 ⁻⁶	S	E31	10 ⁻⁶	S
E40	10 ⁻⁵	D 2	E45	10 ⁻⁵	S	E33	10 ⁻⁵	S

Each group of C and E rats is a litter. Each E rat was fed the bacteria from 10 cc. growth on each of 2 successive days. Test took place 2 days after 2nd feeding.

It is clear from the data presented in Tables XXII to XXX that the ingestion of the intact dead cell or of the Berkefeld filtrate of the sodium glycocholate-dissolved organism produces an increased resistance to *Pneumococcus* Type III. It also seems as if the dose and the time of appearance of the immunity are approximately the same as for Type I. A single feeding of the bacteria from 5 cc. growth is sufficient and the effect is evident in 48 hours.

In order to learn whether the immunity thus produced is type-specific, rats were fed *Pneumococcus* Type III and tested for resistance to Type I. Others were fed Type I and tested with Type III.²

² Earlier experiments, performed before it was known that rats exhibit a variation in resistance to Type III, indicated that feeding Type I did not protect against Type III.

Experiment 10, Table XXXI.—The Type III organism was grown in glucose meat extract broth. The centrifuged bacteria were suspended in water and mixed with cracker meal. 1 of 2 rats injected with 10^{-8} cc. and 1 of 2 injected with 10^{-6} cc. survived among the treated animals, giving the impression that there was some protection. The experiment was repeated.

TABLE XXXI

Resistance to Pneumococcus Type I, Following Ingestion of Pneumococcus Type III

Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.	
C81	10^{-8}	D 2	E65	10^{-8}	D 3
C61	10^{-8}	D 3	E72	10^{-8}	S
C82	10^{-7}	D 3	E88	10^{-7}	D 2
C84	10^{-7}	D 2	E74	10^{-7}	D 2
C89	10^{-8}	D 3	E101	10^{-8}	D 2
C86	10^{-8}	D 5	E95	10^{-8}	S
C94	10^{-8}	D 2	E101	10^{-8}	D 1

Each E rat received the bacteria from 5 cc. growth, killed with N/12 HCl on each of 2 successive days. Test was done 3 days after 2nd feeding.

TABLE XXXII

Resistance to Pneumococcus Type I, Following Ingestion of Pneumococcus Type III

Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.	
C78	10^{-8}	S	E79	10^{-8}	D 2
*C78	10^{-8}	D 5	E65	10^{-8}	D 2
C81	10^{-8}	D 2	E83	10^{-7}	D 2
C88	10^{-8}	D 2	E80	10^{-7}	D 2
C97	10^{-7}	S	E91	10^{-8}	D 2
C90	10^{-7}	D 2	E90	10^{-8}	D 2
C98	10^{-8}	D 2	E93	10^{-8}	D 2
C104	10^{-8}	D 2	E98	10^{-8}	D 2

Details same as in Table XXXI.

* Pneumococcus in heart blood.

Experiment 11, Table XXXII.—The details are the same as in Experiment 10 except that there were 3 feedings and the test took place 2 days after the last of these. All 8 treated rats succumbed. Among the controls, 1 of 2 animals injected with 10^{-7} cc. survived. It can be seen therefore that very little if any immunity against Type I resulted from the ingestion of Type III organisms.

Experiment 12, Table XXXIII.—Each of 3 litters was divided into controls and experimental rats. The Type I pneumococcus was grown in glucose meat extract

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broth. The survival of the 2 treated rats injected with 10^{-8} and 10^{-7} cc. respectively in the 3rd litter, possibly suggests some cross-protection. In view of the variation in resistance to Type III discussed above, and further illustrated by the survival of both controls (in Litters 1 and 2) injected with 10^{-6} cc. in the present

TABLE XXXIII
Resistance to Pneumococcus Type III, Following Ingestion of Type I

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C49	10^{-8}	D 2	C64	10^{-8}	D 2	C70	10^{-8}	D 2
C50	10^{-7}	D 2	C67	10^{-7}	D 2	C74	10^{-7}	D 2
C50	10^{-6}	S	C72	10^{-6}	S	C74	10^{-6}	D 2
C64	10^{-5}	D 1	C78	10^{-5}	D 1	C81	10^{-5}	D 2
E42	10^{-8}	D 2	E66	10^{-8}	S	E69	10^{-8}	S
E55	10^{-7}	D 2	E68	10^{-7}	D 2	E71	10^{-7}	S
E57	10^{-6}	D 2	E73	10^{-6}	D 2	E72	10^{-6}	D 2
E59	10^{-5}	D 1	E76	10^{-5}	D 1	E83	10^{-5}	D 2

Each E rat was fed the bacteria from 5 cc. growth on each of 2 successive days. Test was done 3 days after 2nd dose. Each group of C and E rats is a litter.

TABLE XXXIV
Resistance to Pneumococcus Type III, Following Ingestion of Type I

Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C35	10^{-8}	S	C32	10^{-8}	D 2	C33	10^{-8}	D 2
C41	10^{-7}	S	C33	10^{-7}	D 1	C33	10^{-7}	D 1
C43	10^{-6}	D 1	C34	10^{-6}	D 1	C33	10^{-6}	D 1
C45	10^{-5}	D 1	C36	10^{-5}	D 1	C38	10^{-5}	D 1
E40	10^{-8}	D 2	E34	10^{-8}	S	E32	10^{-8}	D 2
E40	10^{-7}	S	E34	10^{-7}	D 1	E34	10^{-7}	D 1
E41	10^{-6}	S	E35	10^{-6}	D 1	E34	10^{-6}	D 2
E50	10^{-5}	D 1	E39	10^{-5}	D 1	E39	10^{-5}	D 1

Each E rat received the bacteria from 7 cc. Type I growth, killed by N/12 HCl, on 1 day, followed by 13 cc. the next day, suspended in milk and given by medicine dropper. Test was done 3 days after 2nd dose. Each group of C and E rats is a litter.

experiment, the results are probably to be considered as indicating no cross-protection.

Experiment 13, Table XXXIV.—Each of 3 litters was divided into control and treated rats. The organism was grown in beef heart broth. There were 10 deaths among the controls and 9 among the treated animals, indicating the absence of any appreciable cross-protection.

DISCUSSION

The marked difference in resistance among rats to both Types II and III pneumococci is apparently the reflection of a natural partial immunity. This partial immunity which appears at a considerably earlier age in some animals than in others, and which is quantitatively greater for Type II than Type III, contrasts markedly with the equal and great susceptibility of young and old rats to Type I pneumococcus. No experimental data are available which explain the appearance of this partial immunity toward these types in the rat. It is possible that the rat harbors these types of pneumococcus normally in the same way that many human beings do. It is also conceivable that among its intestinal flora are some microorganisms sufficiently closely related antigenically to Types II and III pneumococci to give protection against them. This necessarily assumes that such organisms are antigenically more closely related to these types of pneumococci than the latter are to Type I, and that they also can immunize by their presence in the intestine.

Avery, Heidelberger and Goebel (4) have found a close immunological relationship between Type II pneumococcus and a certain strain (E) of Friedländer's bacillus extending to reciprocal protection of the antisera, and which is probably based on the marked resemblance, both chemical and immunological, which they demonstrated existed between the purified specific substances of these organisms.³ Sugg and Neill (5) have demonstrated a similar close resemblance between a variety of *Saccharomyces cerevisiae* and Type II pneumococcus as determined by immune serum reactions, and which they thought was based on an S-anti-S relationship. Sugg, Richardson and Neill (6) have actively immunized mice against Type II pneumococcus by vaccination with this yeast. It would seem from these observations that a partial natural immunity to some types of pneumococci by fortuitous infection with a biologically distantly related microorganism is possible.

It is now evident that the successful immunization of rats against

³ The part which the polysaccharide of the pneumococcus plays in oral immunization is discussed in the next paper, as well as in the papers entitled "The rôle of the soluble specific substance in oral immunization against *Pneumococcus* Types II and III," and "The fate of orally administered soluble specific substance of *Pneumococcus* Types I, II and III."

question of whether this substance may be considered the only constituent which is immunologically active when the pneumococcus is fed, that the present report is made. These data are as follows: (1) the similarities between the effects of feeding the pneumococcus and the polysaccharide to rats, (2) comparison of results of feeding the pneumococcus and polysaccharide to mice, (3) the influence of certain treatment of the bile salt-dissolved pneumococcus and of the whole cell, on the immunity created by feeding these materials.

Although principally concerned with the effect of oral administration of the specific substance, experiments are included in which the polysaccharide was injected into mice, because of the bearing these have on the problem as a whole. All the experiments were done with Type I specific substance and Type I pneumococcus.

The Soluble Specific Substance.—Two samples of carefully prepared polysaccharide obtained through the courtesy of Dr. Harry Sobotka,¹ and which will be referred to as Lots 1 and 2, were employed. There was too little of Lot 1 to do extensive chemical tests. The xanthoproteic and Millon tests were negative for both. The biuret gave a faintly positive result when done on relatively large quantities of dry substance of Lot 2. Both samples could be detected in a dilution of 1 part in 3 million of water by the use of antiserum. Analyses² of Lot 2 yielded the following data: nitrogen, 5.1 per cent; carbon, 41.7 per cent; hydrogen, 5.6 per cent; ash, 0.4 per cent; reducing substances in terms of glucose (after hydrolysis), 24.7 per cent. These figures are calculated on the dry basis. There was a loss of 16 per cent on drying at 100°C. The reducing substances were determined by the Shaffer-Hartmann-Somogyi method, after boiling several milligrams with 1 cc. of 1.4 normal nitric acid under a reflux condenser, and neutralizing the mixture. These figures agree fairly well with those of Heideberger, Goebel and Avery (3).

Resemblance of the Effects of Feeding the Soluble Specific Substance to Those Obtained When the Pneumococcus was Orally Administered

The immunizing effect of feeding the pneumococcus has been largely dealt with in earlier papers. Table III in the present report illustrates

¹ He also furnished the Type II and III preparations used in experiments described in the paper, "The rôle of the soluble specific substance in oral immunization against *Pneumococcus* Types II and III," in the following number.

² Analyses for C, H, N, ash and loss on heating, were kindly performed by Dr. Frances Krasnow, of the College of Physicians and Surgeons of Columbia University.

the features which characterize the protection so obtained. These are (1) a single feeding of the bacteria contained in 5 cc. growth is sufficient; (2) the increased resistance appears within 48 hours; (3) in extent it is equivalent to 1000 to 10,000 fatal doses;³ (4) it lasts for about 2 weeks at which time a new feeding causes it to reappear. This can be repeated as often as one wishes. The duration of 2 weeks refers to the experiments in which 1 or more feedings of the bacteria from 5 cc. growth are given. By increasing the size of each dose the immunity can be made to last for longer periods of time.⁴

Number of Feedings Required. Degree and Time of Appearance of Immunity.—In the first experiment each animal received 0.5 mg. (Lot 1) on each of 3 days. The material was dissolved in N/15 HCl. The solution was mixed with cracker meal and each rat was fed individually. All tests for measuring the resistance to pneumococcus in this and subsequent experiments were done by injecting the required dose intraperitoneally in a volume of 0.2 cc. It will be seen from Part 1 of Table I that the rats which were fed the specific polysaccharide possess an increased resistance to the virulent organism. Among the controls, 10^{-8} and 10^{-7} cc. killed, in each case, 2 out of 3 rats. Among the treated rats, 2 injected with 10^{-8} cc., 2 with 10^{-7} cc. and 1 with 10^{-5} cc. survived. The one injected with 10^{-6} cc. died.

In the next experiment the number of feedings (Lot 1) was reduced to 2. The procedure was the same as described above, except that a slight insoluble residue was separated from the solution by centrifugation (Part 2, Table I). Although the response is not quite uniform, some rats are protected against 1000 fatal doses. 1 of 2 survived 1 fatal dose, 1 of 2 lived after receiving 10 fatal doses, 2 of 3 survived 100 and 1000 fatal doses, respectively.

The number of feedings was then further reduced to 1 (Lot 1), keeping the conditions the same as they were in the preceding experiment. The result is shown in Part 3 of Table I. Although the extent of resistance to the pneumococcus reaches 1000 fatal doses in some instances, a smaller proportion of animals was protected than when 2 or 3 doses were administered.

In Part 1 of Table II are given the results of an experiment in which the Type I specific substance of Lot 2 was fed. Two solutions were prepared, one in which the material was dissolved in N/15 HCl; in the other the solvent was a phosphate solution of pH 7.7. The slight insoluble residue in both was removed. The HCl solution was fed to one lot of rats and the phosphate solution to another, with the object of learning whether the solvent exerted any influence. There were 2 feedings. The results indicate that the two solvents gave about the same general

³ Less in the case of Types II and III.

⁴ See, for example, Tables IV and V in Reference 1.

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animals killed by the pneumococcus lasts for at least 4 months, at which time it is almost, if not quite as high as it is directly after the feeding (4). Although the protection against the pneumococcus which follows the oral administration of 1 dose of acid-killed organisms from 5 cc. growth is quantitatively as high as is obtained when repeated doses of infected tissue are fed, its duration appears to be considerably less.⁶ It is the similarity between the duration obtained in the former kind of experiment and that which follows feeding of the soluble

TABLE IV
Duration and Renewal of Immunity Produced by Feeding Soluble Specific Substance

Dose cc.	48 hrs. after 2nd of 2 feedings		10 days after 2nd of 2 feedings		72 hrs. after a new feeding	
	C	E ^a	C	E ^b	C	E ^c
10 ⁻⁹	S.S.S. 2					
10 ⁻⁸	2.2.5.4		S.S.	S.S		
10 ⁻⁷	2.2	S.2	S.S	S.S.2	7*	
10 ⁻⁶	2.	3.3.S	S.S	3.3.2	8*.2	S.S.S
10 ⁻⁵	3.	3.2.3	2.5	2.2	2.3	S.S.S.4*
10 ⁻⁴		S.S.2	2.	2.2	5.3	S.S. 7†
		2.2		2	2.	S.2.4
						2.2

E^a and E^b rats received 0.5 mg. S.S.S. (Lot 2) on each of 2 successive days. S.S.S. was dissolved in N/15 HCl, centrifuged and filtered through filter paper. E^c rats received same 2 feedings and in addition 0.5 mg. dissolved in N/15 HCl, centrifuged but not filtered, 12 days after 2nd feeding.

* Contaminant in heart blood. † Pneumococcus in heart blood.

⁶ Did not receive new feeding; had a litter after 2nd feeding.

specific substance, that is brought out by the following observations. Table III shows what happens when 1 dose of acid-killed pneumococci is given.

Two experiments with the specific polysaccharide were done.

The results of the first are given in Table IV. Each animal received 0.5 mg. (Lot 2) on each of 2 successive days. 2 days after the 2nd dose some of the rats were tested and it was found that, of 13 rats, 4 had survived quantities of pneumococci equivalent to from 1 to 1000 fatal doses. It seemed that the low percentage of survivors might make it difficult to determine the disappearance of the protection, but judging by the data of Column 2 of the same table, (13 additional rats of the original group) it is evident that most, if not all of the increased resistance, was lost 10 days after the feedings. For some reason the controls survived somewhat

⁶ See Tables IV and V, Reference 1.

larger doses than the experimental rats.⁷ The remaining animals of the original group were now fed 1 additional dose of 0.5 mg. of specific substance (Lot 2) and were tested 3 days later, with the result shown in the third column of this table. It is evident that the protection is renewed. More uniform results were obtained than after the first feedings.

The experiment was repeated, using Lot 1 of the soluble specific substance. The data appear in Table V. The immunity which followed the ingestion of 2 doses of polysaccharide appeared in 48 hours and was as regular and as high as that which follows the ingestion of the intact acid-killed pneumococcus or the bile salt-

TABLE V

Duration and Renewal of Immunity Produced by Feeding Soluble Specific Substance

Dose	48 hrs. after 2nd of 2 feedings		10 days after 2nd of 2 feedings		72 hrs. after a new feeding	
	C ^a	E ^a	C ^b	E ^b	C ^c	E ^c
<i>cc.</i>						
10 ⁻³	3*.2		S.2*	S.S	S.S	
10 ⁻⁵	S.2	S.S	2.2	S.2	2*.3	2 ¹
10 ⁻⁷	2.2	S.S.S	2.2	S.S.2	3.3	2 ¹ .S ²
10 ⁻⁹	2.1	S.S.4	2.2	2.2	3.2	S ⁴ .3 ⁴ .S ²
10 ⁻¹	2.	S.S.S	2	2.2	3	2 ¹ .S ⁷ .2 ³

The E^a and E^b rats were fed 0.5 mg. S.S.S. (Lot 1) on each of 2 successive days. The S.S.S. was dissolved in N/15 HCl, centrifuged and the supernatant fluid was mixed with cracker meal. The E^c rats received the same 2 feedings and in addition 0.5 mg. S.S.S. similarly prepared, 19 days after the 2nd feeding. C^a and C^b were fed N/15 HCl alone in cracker meal.

The exponents in the E^c column identify the rats in Table II, Experiment 1, in the paper "Fate of orally administered soluble specific substance of Pneumococcus Types I, II and III" in the following number.

* Pneumococcus in heart blood.

dissolved cell. 10 days after the 2nd dose some additional rats were tested. A smaller percentage of animals was protected and the general appearance of the results is like that which is found after a similar interval of time when 2 doses of the dead bacteria are fed. The remaining untested animals were fed 0.5 mg. each of the polysaccharide and were injected with the pneumococcus 72 hours later. The original immunity reappears but, as in most of the other experiments with the soluble specific substance, a single feeding seems to be insufficient to give the regularity of protection that a single ingestion of the organisms yields.

The immunity created by the oral administration of the specific substance to white rats is thus seen to bear a marked resemblance to

⁷ The C and E rats came from different sources. However, this has rarely been found to be correlated with a difference in the minimum fatal dose.

that obtained by feeding small quantities of the organism with respect to duration as well. The data below show the possibility that there is a second active substance within the pneumococcus cell.

*Comparison of Effects of Feeding the Pneumococcus and the Soluble Specific Substance, Using Mice as Experimental Animals.
Injection of the Soluble Specific Substance*

The following experiments were done with the object of learning whether the specific substance plays as important a part in the oral immunization of mice as it does in the rat. It has been stated in an earlier communication (5) that mice are not well immunized by feeding the pneumococcus. A smaller percentage of animals is protected than when rats are used, and in some experiments no evidence of any success is obtained. This made it apparent from the start that the rôle of the polysaccharide in the oral immunization of this animal would be more difficult to determine than in the case of rats. Aside from this, however, if mice could be protected against the pneumococcus by feeding the soluble specific substance with approximately the same degree of success as when the bacteria are used, there would be evidence that it is equally important in the oral immunization of both animals. On the other hand a failure to protect by feeding the polysaccharide might mean that a constituent other than this is active when mice are fed the intact or dissolved cocci.

Feeding the Intact Pneumococcus and the Bile Salt-Dissolved Cell to Mice.—In Table VI are given the data of an experiment in which acid-killed pneumococci were administered. Of 7 controls injected with 10^{-8} cc., 1 lived. Among the treated mice, 1 of 3 survived 10^{-8} cc., 5 of 10 survived 10^{-7} cc. and 5 of 8 survived 10^{-6} cc. The response is inferior to that obtained in rats. No difference in results which might be ascribed to the difference in initial weights of the mice (11 to 25 gm.) was observed.

Table VII gives the data for a similar experiment in which acid-killed and heat-killed pneumococci were used. The moderate degree of protection that was found in the preceding test was not obtained. Counting only the mice which were fed the HCl-killed cells,⁸ 2 survived 1 fatal dose, 1 lived after receiving 10 and 200 fatal

⁸ Since heating at 80°C. for 2 hours has been found to largely destroy the immunizing action for rats, the same would probably be expected for mice. The presence of a little acid from the culture doubtless played a part in the loss which allowed such heating.

doses, respectively. This, however, takes no account of the mice in the last column of the table where treated animals succumbed to doses of 10^{-8} and 10^{-7} cc., whereas 1 control injected with 10^{-8} cc. lived, and 1 of 2 injected with 10^{-7} cc. also sur-

TABLE VI

Resistance of Mice to Pneumococcus Following Feeding of Acid-Killed Organisms

Dose	C	E 15/2	C	E 17/1	C	E 17/2	C	E 17/7	C	E 17/9	C	E 17/12
cc.												
10^{-9}			S									
10^{-8}	2	S	2	4	3	2	2.2		S	2		
10^{-7}	2	S	2	S	2	2	2	S S	2	2.2	2	S.3.2
10^{-6}	2	2	2	S	1	2	2	S.S	2	3	2	S.S
10^{-5}										2		

C = control. E = treated mouse. S = survived. Number = died, — days.

The figures at the head of the E columns give the number of feedings, followed by the interval in days between the last feeding and the day of the test. Growth was killed by 2 hours contact with N/15 HCl at room temperature. Mice weighed between 11 and 25 gm. at the beginning of the experiment.

TABLE VII

Resistance of Mice to Pneumococcus Following Feeding of Acid- and Heat-Killed Organisms

Dose	C	$\frac{3}{E^h E^a}$		C	$\frac{4}{E^h E^a}$		C	$\frac{6}{E^h E^a}$		C	$\frac{11}{E^h E^a}$		C	$\frac{14}{E^h E^a}$		C	$\frac{18}{E^h E^a}$	
cc.																		
10^{-9}	S			S						S			S					
10^{-8}	S	S	3	2	2	S	2	S	3	S	S	2	2	3	S	S	2	4.2
2.10^{-7}	2	2	6															
10^{-7}				3	2	S	2	2	2	2	2	2	2	2.3	2.2	S.2	2.2.2	2.2.2
2.10^{-6}							2	2	2									
10^{-6}				2	2	2				2	2	2	2	2.2	2.2	2	6	2.2
10^{-5}													2	2	2			

E^h and E^a mice fed heat-killed and acid-killed organisms respectively. The figure above E^h and E^a equals the interval in days between the last of 16 feedings (over a period of 25 days) and the day of test. For killing by acid, 2 hours contact with N/15 HCl was used. For killing by heat, concentrated suspensions of pneumococci were heated 2 hours at 80°C . Mice weighed 20 gm. or more at beginning of experiment.

vived. Also in the tests done 3 and 11 days after feeding, the controls survived 10^{-8} cc. while the rats fed the acid-killed bacteria succumbed.

Because the vaccine employed had been stored (and used as needed) it seemed that the indecisive results might be owing to a destruction of the antigen. Hence the test was repeated and the bacteria were grown daily. Three forms of pneumo-

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cocci were employed: living, acid-killed and heat-killed (80°C. for 2 hours). The mice in each of these three groups were subdivided into those weighing between 7 and 10 gm. and those whose weights were between 18 and 30 gm. The data are given in Table VIII. Again excluding from the count those animals fed the heat-

TABLE VIII
Resistance of Mice to Pneumococcus Following Feeding of Living, Acid-Killed and Heat-Killed Organisms

Dose	C	7			C	11			C	13
		E ¹	E ^a	E ^b		E ¹	E ^a	E ^b		E ¹
cc.										
10 ⁻⁹	S				S				S	
10 ⁻⁸	2	2.2	2	2	2.5	2.S.S.S.S.	2.2.S.		S	2.2.S.
10 ⁻⁷	2	4		2	2	S.2.2.2.5.4.	2.2.S.	S.S.	S	3.2.2
10 ⁻⁶	2	2.3	2	2	2	2.2.2.3.	2.1.2	2.2.2.	2	S.2

E¹, E^a and E^b = mice fed living, acid-killed and heat-killed organisms respectively.

Figures above these letters = intervals in days between last of 17 feedings of living organisms (over a period of 28 days), and day of test, and interval between last of 12 feedings of heat- or acid-killed (over a period of 25 days) and day of test.

TABLE IX
Resistance of Mice to Pneumococcus Following Feeding of Acid-Killed Organisms

Dose	C	E ^a	C	E ^b	C	E ^c
cc.						
10 ⁻⁹						
10 ⁻⁸	2.2	3.S	S.S.S.			
10 ⁻⁷	2.2	4.S	2.2.2.		6.2*	
10 ⁻⁶	2.2	2.S	2.2	S.S	2.2	2
10 ⁻⁵			2.2	5.3.4.	2.2	3.3
				S.3.2.	2.2	2.S
				5		

E^a and E^b mice each received bacteria from 50 cc. growth each of 5 days (over a period of 6 days). E^c mice received 3 feedings (organisms from 30, 30 and 50 cc. on 3 successive days). The first feeding in all cases was preceded by a 24 or 48 hour fast. Test took place 7 days after last dose for E^a and E^b mice, and 11 days after last dose for E^c mice.

* Pneumococcus in heart blood.

killed organisms, we find among those given either the living⁹ or acid-killed pneumococci, the following: of 11 injected with 10⁻⁸ cc., on days when this was a fatal dose for controls, 5 lived; of 10 injected with 10⁻⁷ cc., 2 lived. The only other larger dose used, 10⁻⁶ cc., killed all the treated animals. This does not take

⁹ The feeding of living pneumococci caused many deaths among the mice but none among the rats.

TABLE X

Resistance of Mice to *Pneumococcus* Following Feeding of Organisms Grown in Milk
or Dissolved in Sodium Taurocholate

Weight	Dose	Result	Feedings and interval	Remarks	Weight	Dose	Result	Feedings and interval	Remarks
gm.	cc.				gm.	cc.			
C 20	10 ⁻⁸	S		n.d.	C 21	10 ⁻⁸	3		n.d.
C 22	10 ⁻⁷	2		n.d.	C 20	10 ⁻⁸	2		s.t.
C 23	10 ⁻⁸	3		n.d.	C 20	10 ⁻⁷	2		s.t.
E 18	10 ⁻⁸	4	18/1	Milk	C 23	10 ⁻⁸	2		s.t.
E 19	10 ⁻⁷	2	18/1	Milk	E 19	10 ⁻⁸	6	18/1	T.I
					E 20	10 ⁻⁷	3	18/1	T.I
					E 20	10 ⁻⁸	2	18/1	T.I
C 20	10 ⁻⁸	2		n.d.	E 18	10 ⁻⁸	2	9/1	Milk
C 22	10 ⁻⁷	2		n.d.	E 21	10 ⁻⁷	2	18/1	Milk
C 22	10 ⁻⁸	2		n.d.	E 23	10 ⁻⁸	2	9/1	Milk
E 22	10 ⁻⁸	2	19/2	Milk					
E 17	10 ⁻⁸	S	19/2	Milk	C 19	10 ⁻⁸	2		n.d.
E 22	10 ⁻⁷	2	19/2	Milk	C 20	10 ⁻⁷	2		n.d.
E 22	10 ⁻⁷	2	19/2	Milk	C 23	10 ⁻⁸	2		n.d.
E 22	10 ⁻⁸	2	19/2	Milk	E 19	10 ⁻⁸	2	19/2	T.I
E 23	10 ⁻⁸	2	19/2	Milk	E 22	10 ⁻⁷	S	19/2	T.I
					E 23	10 ⁻⁸	S	19/2	T.I
C 21	10 ⁻⁷	2		n.d.	E 20	10 ⁻⁸	S	10/2	Milk
C 22	10 ⁻⁸	2		n.d.	E 21	10 ⁻⁷	2	19/2	Milk
C 19	10 ⁻⁷	2		s.t.	E 23	10 ⁻⁸	3	10/2	Milk
C 20	10 ⁻⁸	2		s.t.					
E 19	10 ⁻⁷	S	13/1	T.I	C 22	10 ⁻⁸	2		n.d.
E 20	10 ⁻⁸	4	13/1	T.I	C 23	10 ⁻⁷	2		s.t.
E 20	10 ⁻⁷	S	16/1	Milk	C 27	10 ⁻⁸	2		n.d.
E 21	10 ⁻⁸	2	16/1	Milk	E 23	10 ⁻⁷	3	19/3	T.I
					E 26	10 ⁻⁸	S	19/3	T.I
C 20	10 ⁻⁸	3		n.d.	E 23	10 ⁻⁸	S	19/3	Milk
C 20	10 ⁻⁷	S		n.d.	E 23	10 ⁻⁷	4	19/3	Milk
C 21	10 ⁻⁸	4		n.d.	E 24	10 ⁻⁸	2	19/3	Milk
C 20	10 ⁻⁸	2		s.t.					
C 21	10 ⁻⁷	3		s.t.	C 21	10 ⁻⁸	3		n.d.
C 21	10 ⁻⁸	2		s.t.	C 24	10 ⁻⁷	2		n.d.
E 20	10 ⁻⁸	S	17/1	T.I	C 24	10 ⁻⁸	2		n.d.
E 20	10 ⁻⁷	4	17/1	T.I	*E 24	10 ⁻⁷	1	19/4	T.I
E 19	10 ⁻⁸	3	17/1	T.I	E 25	10 ⁻⁷	S	19/4	T.I
E 22	10 ⁻⁸	S	17/1	Milk	E 19	10 ⁻⁸	S	10/4	Milk
E 21	10 ⁻⁷	2	17/1	Milk	E 24	10 ⁻⁷	2	19/4	Milk
E 21	10 ⁻⁸	2	17/1	Milk	E 26	10 ⁻⁸	2	19/4	Milk
					E 28	10 ⁻⁸	2	10/4	Milk
C 19	10 ⁻⁸	2		n.d.					
C 21	10 ⁻⁷	3		n.d.					

The pneumococci were centrifuged and removed with a small quantity of water, rubbed about until evenly distributed, sodium taurocholate added, and left for about 1 hour to dissolve the organisms. Microscopic examination of stained smears were made daily to observe the solvent effect; only an occasional intact cell was observed. The mice were not fed individually, but the dissolved pneumococci, soaked up in bread, and the cultured milk, were put in dishes into the cages. In the case of the bile salt-dissolved organisms, the amount fed was equivalent to 20 cc., per mouse (average). The quantity of sodium taurocholate employed was equivalent to 2 to 3 mg. per mouse per day, and the controls received this as well. The cultured milk was fed in quantities averaging 2 cc. per mouse daily.

Milk = fed cultured milk. s.t. = fed sodium taurocholate alone. n.d. = nothing done. T.I = fed sodium taurocholate-dissolved *Pneumococcus* Type I.

* Sick before injection.

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account of the last day's test in which 10^{-7} cc. was the fatal dose for a control, whereas 2 of 3 treated mice succumbed to 10^{-8} cc., all 3 injected with 10^{-7} cc., and 1 or 2 injected with 10^{-6} cc. died.

In Table IX are listed the data of an experiment in which the ingestion of acid-killed pneumococci by mice was preceded by a period of fasting and accompanied

TABLE XI
Resistance to Pneumococcus of Mice Kept on an Exclusive Milk or Meat Diet and Fed Berkefeld Filtrate of Sodium Glycocholate-Dissolved Organisms

Dose cc.	C	5 days after last dose		7 days after last dose		
		E ^a	E ^b	C	E ^a	E ^b
10^{-9}	S.S			5.S		
10^{-8}	2.3			2.2		
10^{-7}	2.2	4.S	2.2	2.2	2.2	2
10^{-6}	2.2	7.S	3.2	2.2	S.2.2	2.2.2
10^{-5}	2.2	2.S	2.3	2.2	5.2.2	2.2.2

E^a mice kept on milk diet. E^b mice kept on meat diet. Each E mouse received the bacteria from 2, 5 and 5 cc. on 3 days (over a period of 5 days).

TABLE XII
Resistance of Mice to Pneumococcus Following Feeding of HCl-Killed Mechanically Disrupted Organisms

Dose cc.	C	5 E	C	6 E	C	9 E	C	11 E	C	12 E	C	15 E
10^{-8}	2	2	3	3	2	2.3	2	S	2	S	3	3
10^{-7}	2	3	3	8	2	3.2	2	3.2	3	2	3	3.3
10^{-6}	2	2	2	2	3	2.2	2	3.3.3	2	2	2	3.3

The figure above E equals the interval in days between last dose and test. The E mice were fed pneumococcus, grown in glucose meat extract broth, pH 7.6, killed by HCl and desiccated in partial vacuum. The pulverized bacteria were mixed with milk and fed by medicine dropper. A fast of 4 hours preceded and followed each feeding. There were 15 feedings (over a period of 17 days). The bacteria from 30 cc. growth comprised a single feeding for a mouse.

by a restricted diet. Out of a total of 20 treated animals, 3 survived 1 fatal dose, 1 survived 10, 2 lived after injection with 100, and 1 after 1000 fatal doses. The use of bile salt-dissolved organisms and of pneumococci grown in milk, having been found effective for rats, was tried with mice. The number of feedings varied between 9 and 19, and the individual dose from 2 cc. in the case

of the organisms cultured in milk, to 20 cc. for the dissolved cells. A summary of Table X shows that of 31 control mice, 2 survived; of 15 mice fed the dissolved pneumococci, 6 survived, and of 26 fed the cultured milk, 6 lived.

An additional experiment (Table XI) was done with bile salt-dissolved cells, keeping one lot of mice on an exclusive milk, and another on an exclusive meat diet. Of the 14 mice on the milk diet, 1 survived a single fatal dose, 2 survived 10, and 1 lived after 100 fatal doses. None among the meat-fed animals lived.

The ingestion of the desiccated mechanically disrupted pneumococci protected only 2 mice against 1 fatal dose, out of a total of 26 animals (Table XII).

The irregularity of these results with mice, combined with the relatively low percentage of animals protected, indicated in advance that feeding the specific substance would probably yield at best an even smaller proportion of immunized animals.

TABLE XIII

Resistance of Mice to Pneumococcus Following Feeding of Soluble Specific Substance

Dose	Controls	0.20 mg. S.S.S. per mouse on each of 2 days	0.01 mg. S.S.S. per mouse on each of 2 days	0.001 mg. S.S.S. per mouse on each of 2 days
cc.				
10 ⁻⁴	S.S.S.2	S.S.S.2	S.S.2.2.	S.S.S.2
10 ⁻⁵	S.2.2.3	2.2.2.2	S.S.2.2.	2.2.2.2
10 ⁻⁷	2.2.2.2	2.2.2.2	2.2.2.	2.2.2.
10 ⁻⁸	2.2.2	2.2.2	2.2.2	2.2.2

The S.S.S. (Lot 2) was dissolved in HCl, mixed with cracker meal and fed on 2 successive days. Test was done 48 hours after 2nd feeding.

Feeding the Soluble Specific Substance to Mice.—Three experiments were done. In the first, the mice were divided into three groups. Each of the members of one of these received 0.20 mg. (Lot 2) on each of 2 successive days. Those in the second group were fed 0.01 mg. and those in the third, 0.001 mg. each, on each of 2 successive days. Table XIII gives the results. There is no evidence for any immunity under the conditions employed.

Another experiment was done in order to see whether better results might follow the use of intervals between the feedings. The data obtained contain no evidence of any immunity (Table XIV).

It was then decided to use a more prolonged period of feeding. The mice were given 12 doses, each equal to 0.10 mg. per animal. Out of a total of 17 mice so treated, and injected with 1 or more fatal doses, 2 survived; 1 of these had received 1, and the other 10 fatal doses of pneumococci (Table XV).

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It appears from these three experiments that no definite immunity against the pneumococcus followed the ingestion of the soluble specific substance. This is in contrast to the protective effect produced in rats by this means, and would seem to suggest that there is a second substance within the cell responsible for the protection created in some mice as a result of feeding the pneumococcus. This possibility will be

TABLE XIV
Resistance of Mice to Pneumococcus Following Feeding of Soluble Specific Substance

Dose	Controls	E ^a	E ^b	E ^c
cc.				
10 ⁻⁹	S.S.S.2	S.S.1	S.2.2	S.S.S
10 ⁻⁸	S.2.2.3	S.S.2	2.2.2	S.2.3
10 ⁻⁷	2.2.2.2	2.2.2	2.2.3	2.2.2
10 ⁻⁶	2.2	2.2.2	2.2.1	2.2.2
10 ⁻⁵		1.2.2	2.2.1	1.2

There were 3 feedings of S.S.S. (Lot 2) dissolved in HCl. Interval between 1st and 2nd feedings, 3 days; between 2nd and 3rd, 4 days. Test took place 3 days after last dose. Quantities fed:—E^a, 0.001, 0.01 and 0.02 mg. E^b, 0.01, 0.01 and 0.02 mg. E^c, 0.10, 0.10 and 0.10 mg.

TABLE XV
Resistance of Mice to Pneumococcus Following Feeding of Soluble Specific Substance

Dose	Controls	E
cc.		
10 ⁻⁹	S	S.S.S
10 ⁻⁸	S.S.S	S.S.S.S.
10 ⁻⁷	2.2	S.2.2.2.2.2
10 ⁻⁶	2.2	S.2.2.2.2.2
10 ⁻⁵		2.2.2.2.2

There were 12 feedings (over a period of 16 days). S.S.S. (Lot 2) was dissolved in HCl. Each feeding = 0.1 mg. per mouse. Test took place 2 days after last feeding.

discussed later. The failure to immunize cannot be owing to a destruction of the soluble specific substance in the intestine, because a similar effect would be expected to follow the ingestion of the whole or dissolved organism. Furthermore, a large porportion of orally administered polysaccharide can be recovered from the feces. Since the bile salt-dissolved pneumococcus protected 6 out of 15 mice, the need

for an intact cell is obviously ruled out. Insufficient dosage does not seem to be the cause since 1.2 mg. was fed to each mouse in the final feeding experiment, and this amount is probably greater than is contained in the bacteria fed to those mice which were immunized by this means.¹⁰

Injection of the Soluble Specific Substance.—In order to see whether the soluble specific substance would produce an immunity in mice when given parenterally, several experiments were done in which the animals were injected either subcu-

TABLE XVI

Resistance of Mice to Pneumococcus Following Subcutaneous or Intraperitoneal Injection of Soluble Specific Substance

Dose	Part 1			Part 2	
	C	Subcutaneous injection		C	Intraperitoneal injection
		0.1 mg. per mouse	0.01 mg. per mouse		0.1 mg. per mouse on each of 3 successive days
cc.					
10 ⁻¹	S.2	S.S.3	S.2.2	S.S.3*	S.S.S.S
10 ⁻²	2.	2.4.2	2.2.2	2.2.3	2.2.3.2
10 ⁻³	2.2	2.2.2	2.2.2	2.2	S.2.2.3
10 ⁻⁴	2	2.2.2	2.2.2	2.2	S.2.2.2
10 ⁻⁵		2.2.2	2.2.2		2
10 ⁻⁶		2.2.2	2.2.2		
10 ⁻⁷		2.2.2	2.2.2		

S.S.S. (Lot 2) dissolved in phosphate solution pH 7.73 and filtered through a Berkefeld V candle. 0.20 cc. injected for the 0.1 mg. doses. For mice getting 0.01 mg., the solution was diluted ten times with phosphate solution. Test was done 72 hours after the final injection of S.S.S. The 2 experiments were done at different times.

* Contaminant in heart blood.

taneously or intraperitoneally. The results are given in Tables XVI and XVII. In Table XVI, Part 1, the data of an experiment in which mice were subcutaneously injected with a single dose of the polysaccharide, appear (Lot 2). Each animal received either 0.01 or 0.10 mg. and 3 days later was tested. There is no sign of immunity among the treated animals.

A similar test in which the polysaccharide (Lot 2) was injected intraperitoneally, is summarized in Part 2 of Table XVI. Each mouse received 3 equal doses.

¹⁰ Judging by the yield of 2.75 mg. of S.S.S. recovered from the growth from 1 l. of Type II culture obtained by Heidelberger and Avery (6).

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There were 2 survivors; 1 had been injected with 10, the other with 100 fatal doses.

To see whether starting with a small dose and increasing its size would give better results, mice were injected subcutaneously with 0.001, 0.02 and 0.10 mg., the intervals being 3 days between the 1st and 2nd, and 4 days between the 2nd and 3rd injections. Another group received the same doses, at the same time, intraperitoneally. The control mice were injected with phosphate solution alone.¹¹ The data are shown in Table XVII, Part 1. Considering 10^{-8} cc. as a minimum fatal dose, 1 of 6 controls and 4 of 8 treated mice survived 1 such dose, 1 treated animal survived 10, 100 and 1000 fatal doses respectively.

TABLE XVII
Resistance of Mice to Pneumococcus Following Subcutaneous or Intraperitoneal Injection of Soluble Specific Substance

Dose cc.	Part 1			Part 2		
	Controls	S.S.S. Subcut.	S.S.S. i.p.	Controls	S.S.S. Subcut.	S.S.S. i.p.
10^{-9}	S.S.S.S.S.2	S.2.2	S.S.S.S.	S.3.7	S.S.S.S.	S.S.2.2.7
10^{-8}	2.4.2.S.3.2	S.S.S.2	S.2.2.4	S.2.2.2	S.S.2.2	S.2.2.2.2.2
10^{-7}	2.2.2.2	2.2.2	S.2.3.2	2.2.2.1	2.2.2.2	2.1.5.2.2
10^{-6}	2.2	2.2.2	S.2.2.2	2.2.2.2	S.2.2.2.2	2.2.2.2.3.2
10^{-5}		S.1.2	2.2.2	2.2.2.2	2.2.2.2	2.2.2.2

S.S.S. (Lot 1) was dissolved in phosphate solution, pH 7.5 and filtered through Berkefeld V candle. Dilutions of this were made with more phosphate solution. 1st dose = 0.001 mg., 2nd = 0.02 mg., 3rd = 0.10. Test was done 3 days after last dose. Half the controls received 3 injections of filtered phosphate solution alone, subcutaneously, the other half, intraperitoneally. The experiments (Parts 1 and 2) were done at different times.

The entire experiment was repeated in an identical manner, with the results shown in Part 2 of Table XVII. There is no indication, this time, of any increased resistance to the pneumococcus.

It would seem from these experiments that the injected purified specific substance produced at best only a doubtful immunity in mice. This result further eliminates the destruction of the soluble specific substance in the gastrointestinal tract as the cause of failure to immunize when it is administered to mice orally.

In addition to the possibility that a constituent of the cell other

¹¹ It is interesting to note that the injection of phosphate solution or of weak HCl solution produced an increased resistance to Type I in some rats. Even water did this occasionally.

than the polysaccharide is the active one when the bacteria are fed to mice, it is also conceivable that during isolation and purification, the specific substance undergoes an intramolecular change which does not affect its precipitin reaction but which does influence its antigenic activity. Schiemann and Casper (7) immunized mice by injecting them with bile salt solutions of pneumococci from which all protein had been removed, and which gave a good precipitin reaction for the soluble specific substance. They believed the effect was due to this substance. One may suppose that in such a solution the specific polysaccharide had not been altered in the suggested manner.

These two alternatives, the presence of a second constituent, and a change in the intramolecular arrangement of the polysaccharide, also suggest themselves as possible explanations for the decline in immunizing power for rats, which a bile salt solution of pneumococcus was observed to undergo on standing. Since this experiment is of importance in the consideration of whether a second substance is immunologically active when the pneumococcus is ingested, the data are given here.

Decline in Immunizing Action of a Bile Salt Solution of Pneumococcus on Standing

10 l. of pneumococcus culture were prepared in beef heart broth, pH 7.6 to 7.7. The centrifuged bacteria were dissolved in 500 mg. of sodium glycocholate, and water was added to make 40 cc. After standing and centrifugation, the supernatant was removed and the sediment extracted with 50 cc. water. The combined liquids were passed through a Berkefeld candle. The filtrate, the pH of which was 6.6, was diluted with ten volumes water. The pH was now 6.8.¹² 2 per cent CHCl_3 was added to 1 portion and other portions were heated either at 60°C. for 1 hour or at 90°C. for 10 minutes. All were left at room temperature and exposed to light. At the end of 2½, 3½, 4½ and 12 months tests were made on rats with the part of the solution to which chloroform had been added.¹³ No test was done directly after preparing the mixture because of the fairly uniform results obtained in earlier experiments. These had shown that the equivalent of 5 cc. growth was sufficient to protect a rat against approximately 1000 to 10,000 fatal doses, 72 hours after ingestion. However, a change had been made in the method of preparation of the solution and significance was attached to this only after the first examination after

¹² The pH values of corresponding solutions of sodium glycocholate alone were 5.8 and 6.0.

¹³ After 4½ months the pH was slightly less than 6.2 and after a year it was 5.7.

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TABLE XVIII
Immunizing Effect of Feeding Bile Salt Solutions of Pneumococcus after Standing in Slightly Acid Solution at Room Temperature and Exposed to Light

Directly after preparing			After 2½ mos.			After 3½ mos.			After 4½ mos.			After 12 mos.					
Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.		gm.	cc.		gm.	cc.		gm.	cc.	
C86	10 ⁻⁹	S	C58	10 ⁻⁸	S	C78	10 ⁻⁸	S	C101	10 ⁻⁹	S	C74	10 ⁻⁹	S	C68	10 ⁻⁹	S
*C83	10 ⁻⁹	D3	C68	10 ⁻⁷	S	C80	10 ⁻⁸	D2	C127	10 ⁻⁹	S	C84	10 ⁻⁹	D2	C74	10 ⁻⁹	S
C86	10 ⁻⁸	D2	C88	10 ⁻⁶	D2	C85	10 ⁻⁷	D3	C116	10 ⁻⁸	S	C92	10 ⁻⁸	D2	C77	10 ⁻⁸	D2
C86	10 ⁻⁸	D2				C87	10 ⁻⁷	D2	C141	10 ⁻⁸	D5	C90	10 ⁻⁸	D2	C75	10 ⁻⁸	D2
C97	10 ⁻⁷	D3				C91	10 ⁻⁶	D2	C129	10 ⁻⁸	D3	C93	10 ⁻⁷	D2	C78	10 ⁻⁷	D2
C88	10 ⁻⁷	D2				C95	10 ⁻⁶	D2	C146	10 ⁻⁷	D2	C94	10 ⁻⁷	D2	C98	10 ⁻⁷	D2
C103	10 ⁻⁶	D2							C150	10 ⁻⁷	D3	C95	10 ⁻⁶	D2	C120	10 ⁻⁶	D2
C118	10 ⁻⁵	D4							C155	10 ⁻⁶	D3	C133	10 ⁻⁶	D2	C123	10 ⁻⁶	D2
									C160	10 ⁻⁶	D3	C138	10 ⁻⁵	D2	C125	10 ⁻⁵	D1

Each rat fed equivalent of 5 cc.

E91	10 ⁻⁸	S	E61	10 ⁻⁷	S	E73	10 ⁻⁷	S	E90	10 ⁻⁷	D5	E85	10 ⁻⁸	D2	*E164	10 ⁻⁸	D4
E91	10 ⁻⁸	S	E67	10 ⁻⁶	D3	E80	10 ⁻⁸	S	E58	10 ⁻⁷	D2	E85	10 ⁻⁸	D6	E72	10 ⁻⁸	D2
E96	10 ⁻⁷	S	E69	10 ⁻⁵	S	E78	10 ⁻⁸	S	E67	10 ⁻⁶	D2	E91	10 ⁻⁷	D2	E91	10 ⁻⁷	D2
E100	10 ⁻⁷	S	E76	10 ⁻⁴	S	E89	10 ⁻³	D3	E51	10 ⁻⁶	S	E88	10 ⁻⁷	D2	E98	10 ⁻⁶	D2
E100	10 ⁻⁸	S	E79	10 ⁻³	D2	E82	10 ⁻³	S	E72	10 ⁻⁵	D2	E101	10 ⁻⁶	D2	E106	10 ⁻⁶	S
E101	10 ⁻⁶	S				E92	10 ⁻⁴	D4	E70	10 ⁻³	D2	E93	10 ⁻⁶	S	E107	10 ⁻⁵	D1
E105	10 ⁻⁵	D4				E95	10 ⁻⁴	D4	E95	10 ⁻⁴	S	E104	10 ⁻⁵	D2	E115	10 ⁻⁵	D3
E108	10 ⁻⁵	D2				E99	10 ⁻³	S	E90	10 ⁻⁴	D2	E109	10 ⁻⁵	S			
E110	10 ⁻⁴	D2															
E111	10 ⁻³	D2															

Each rat fed equivalent of 1 cc.

E63	10 ⁻⁸	S	E79	10 ⁻⁸	S	E69	10 ⁻⁸	S									
E65	10 ⁻⁷	D3	E80	10 ⁻⁷	D2	E59	10 ⁻⁸	S									
E61	10 ⁻⁶	S	E84	10 ⁻⁷	D2	E67	10 ⁻⁷	D5									
E67	10 ⁻⁵	S	E87	10 ⁻⁶	D3	E80	10 ⁻⁷	D3									
E74	10 ⁻⁴	D2	E91	10 ⁻⁸	D3	E86	10 ⁻⁶	D2									
E75	10 ⁻³	D2	E92	10 ⁻⁵	S	E80	10 ⁻⁵	D2									
			E100	10 ⁻³	D2	E90	10 ⁻⁵	D2									
			E104	10 ⁻⁴	D3												
Test done 72 hrs. after feeding			Test done 72 hrs. after feeding			Test done 48 hrs. after feeding			Test done 48 hrs. after feeding			Test done 48 hrs. after feeding			Test done 96 hrs. after feeding		

* Pneumococcus in heart blood.

2½ months. In all earlier experiments the bile salt-treated bacteria from 5 cc. culture were dissolved in 0.5 cc. water, whereas in the present one approximately 0.05 cc. was used, and only after passing through the Berkefeld candle was this made up to 0.5 cc. The same quantity of bile salt was employed. Although practically all the cells were opened by both procedures, it seemed that the active material might

not have completely dissolved in the smaller volume of water. A new solution of pneumococci, prepared at this time in the same manner as the original, was therefore tested. In Table XVIII appear the data for this new preparation and for the tests done at the end of $2\frac{1}{2}$, $3\frac{1}{2}$, $4\frac{1}{2}$ and 12 months on the original preparation. In all instances a volume equivalent to 5 cc. growth was fed, and in some a volume equivalent to 1 cc. as well. These quantities were used because it has been found that the minimum dose for approximately maximum degree of protection lies between them.

It will be seen that the freshly prepared solution gave a uniform protection, though in degree, it was less than was obtained when 0.5 cc. water was used to dissolve the bile salt-opened cells from 5 cc. growth, being equivalent to between 100 and 1000 fatal doses.¹⁴ At the end of $2\frac{1}{2}$ and $3\frac{1}{2}$ months there is no definite loss of activity in the original solution as compared with these results. However, after $4\frac{1}{2}$ months there is a decline which becomes more pronounced at the end of a year. The results at the expiration of the latter period are in marked contrast to those obtained with a freshly prepared solution.

Since hydrolysis of the polysaccharide would account for the loss in antigenic activity on standing, an attempt was made to learn whether this took place.

A comparison of the intensities of the precipitin reactions in the solutions referred to above, after they had stood in the laboratory exposed to light for 2 years, and in a freshly prepared solution, was made. The new solution was made in as nearly an identical manner with the original as possible and was found to give precipitates so nearly like those given by the original preparations that little or no significant difference could be observed.¹⁵ It must be realized, however, that there is a considerable error involved in such a determination, particularly in view of the fact that the number of organisms may have been unequal in the 2 cultures compared. It may be that hydrolysis to the extent of 50 per cent of the original quantity of the specific polysaccharide took place, without being detected. At this time the original solution to which CHCl_3 had been added was again tested, together with a part of the solution to which nothing was done. The equivalent of 10 cc. instead of 5 cc. growth, however, was fed, and it was found that the rats were approximately as well protected as they were by feeding the smaller quantity of the

¹⁴ See Tables X, XI and XIII, in Reference 1.

¹⁵ Comparisons were made between solutions (1) to which nothing was done, (2) to which chloroform was added, (3) heated at 60°C. for 1 hour, (4) heated at 95°C. for 10 minutes. 1:10 and 1:100 dilutions of the Berkefeld filtrates to which ten volumes of water had previously been added, were used.

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freshly made solution (Table XIX). This shows that the loss of activity, even after 2 years standing probably did not exceed 50 per cent, and since this is within the limits of error of the precipitin tests, it appears that the hydrolysis of the specific polysaccharide as an explanation for the loss, is not ruled out. The small number of rats protected by the solution which had stood for $4\frac{1}{2}$ and 12 months gives the impression that a loss greater than 50 per cent took place. This may be because the quantity fed (equivalent to 5 cc.) is near the lower limit of the amount which immunizes a large percentage of the animals, and when one uses less than this the proportion of animals protected drops sharply.

TABLE XIX
Immunizing Effect of Feeding 2 Year Old Bile Salt Solution of Pneumococcus
The equivalent of 10 cc. growth was fed to each rat

Dose cc.	Controls		Fed solution to which CHCl_3 was added		Fed plain solution	
	Weight gm.	Result	Weight gm.	Result	Weight gm.	Result
10^{-8}	72	S	65	S	55	S
	73*	D 4	62	S	63	S
10^{-7}	77	S	65	S	60	S
	73	D 2	72	D 6	64	S
10^{-6}	78	D 2	67	S	65	S
	80	D 2	66	S	65	S
10^{-5}			77	S	72	S
			80	S	76	S
			73	S	76	D 5
			84	S	73	S
			96	S	77	S
					84	D 10

D = died, — days. S = survived.

* Pneumococcus in heart blood.

Assuming that the estimation of the specific polysaccharide in the 2 year old solution was correct, however, and that there was actually no loss of this substance, the fall in activity of the solution would suggest that the immunizing action of the specific substance was supplemented, in the fresh preparation, by that of another substance, and that it was this other constituent which had been partially or wholly destroyed on standing. According to this explanation the initial content of specific substance in 5 cc. culture would be considered too low to produce the observed immunity by itself, and hence even if it had not been partly destroyed on standing, would continue to remain too low.¹⁶ On the other hand, in view of the direct demonstration that the purified soluble specific substance does immunize

¹⁶ The minimum quantity of purified S.S.S. necessary to immunize rats regularly has not been determined.

rats, the previously mentioned alternative regarding a change in the intramolecular structure of this substance as an explanation for the loss of activity which the solution suffered on standing, seems more significant.

From a practical point of view the use of solutions of pneumococci for oral immunization would appear to be, for the present, limited to relatively freshly made solutions. In distinction to this it has been found that the procedure used in most of the experiments for killing, namely contact with hydrochloric acid, does not appear to cause any loss in activity¹⁷ and that desiccation, a convenient means of keeping the vaccine, is also without effect. These experiments are reported here because the results are what one would expect in view of the demonstration of the important rôle of the specific polysaccharide, and because they supplement the other indirect evidence which points to the fact that this substance is active when rats are immunized by feeding the whole or the dissolved cell. These data show that the degree of protection and the percentage of animals immunized, closely resemble those obtained when freshly centrifuged living pneumococci are fed, and that therefore little or no loss is sustained by the process of killing and drying.

Although previous data had demonstrated that contact for $2\frac{1}{2}$ to 3 hours at room temperature with $N/12$ HCl, as well as desiccation, left the bacteria in a condition capable of immunizing rats, the tests involved the use of such large quantities of organisms that partial destruction of the active material could not readily be observed. In the experiments described below, a single feeding of the bacteria from 5 cc. was employed (in one case 1 cc. as well), since, as mentioned above, this is the smallest quantity which has been found to give uniform immunity equivalent to 1000 to 10,000 fatal doses of pneumococci, 48 to 72 hours after feeding.

Influence of Contact with $N/12$ HCl Solution and Desiccation.—The pneumococcus was grown in glucose meat extract broth, pH 7.6 to 7.8. After the culture had reached room temperature, normal HCl solution was added to make $N/12$ and the whole left for $2\frac{1}{2}$ hours. After centrifugation, a portion of the bacterial sediment was put in the ice box for use 2 days later. The remainder was spread in a thin layer, dried over $CaCl_2$ in an evacuated desiccator and ground up in a mortar.

¹⁷ At least, with respect to degree and time of appearance of the immunity, as well as to the percentage of animals protected.

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Table XX gives the data and shows that treatment with acid, and desiccation, did not affect the degree of immunity obtained nor the percentage of animals protected. They are about the same as result from the ingestion of untreated organisms.

TABLE XX
Immunizing Effect of a Single Feeding of Desiccated Acid-Killed Organisms. (Rats)

Dose cc.	Moist bacteria kept 2 days in ice box. 48 hrs. after feeding			Desiccated bacteria					
	C rats	E rats fed bacteria from		48 hrs. after feeding			72 hrs. after feeding		
		5 cc.	1 cc.	C rats	E rats fed bacteria from		C rats	E rats fed bacteria from	
					5 cc.*	5 cc.°		5 cc.*	5 cc.°
10 ⁻⁹	S.2								
10 ⁻⁸	2.2								
10 ⁻⁷	2		3.3	S.					
10 ⁻⁶	3	S.S.	S.2	S.3			S.		
10 ⁻⁵		S.S.	S.2	5.	S.S.	S.S.	3.3		
10 ⁻⁴		S.2	S.	3.	S.S.	S.4.	2.6	S.S.	S.S.
10 ⁻³		S.2		4.	S.S.	S.4.	2.5	S.S.	S.S.
		2			3.6	S.4	6	S.3	S.2

* Suspension of bacteria prepared directly before feeding.

° The bacteria were ground up with water in a mortar and left to soak overnight before feeding.

TABLE XXI
Immunizing Effect of a Single Feeding of Pneumococci Kept in Contact with N/12 HCl for 24 Hours at Room Temperature and Then Dried

Dose cc.	24 hrs. after feeding		48 hrs. after feeding		72 hrs. after feeding	
	C	E	C	E	C	E
10 ⁻⁹	S.S	S				
10 ⁻⁸	2.2	S.4	S.S			
10 ⁻⁷	3	3.2	2.2		2.2	
10 ⁻⁶	2	2	2	3	3.2	
10 ⁻⁵		2	1	S.2	3	S
10 ⁻⁴				S.3	2	S.3
10 ⁻³				S		2
				2		3

Each E rat was fed the bacteria from 5 cc. growth.

In another experiment (Table XXI) the culture in glucose meat extract broth was treated with normal HCl to make N/12 and left for 24 hours at room temperature. After centrifugation, the bacterial sediment was left in the desiccator over CaCl₂ at atmospheric pressure for 15 hours, at which time most of it was still wet.

It was then dried in a current of air from an electric fan and powdered. The results, 48 hours after feeding, are somewhat poorer than in the preceding experiment, but 72 hours following the ingestion of the bacteria there is protection against 1000 to 10,000 fatal doses.¹⁸ Little, if any loss resulted from this relatively severe treatment.

The absence of destructive action by the acid on the immunizing substance is in accord with the known resistant behavior of the polysaccharide toward acid at room temperature. Desiccation proves to be, as one would expect, also without effect.

DISCUSSION

Whether or not the soluble specific substance is the only constituent of the Pneumococcus Type I, which is responsible for the immunity which follows the oral administration of this organism to rats, is at present unknown. However, the feeding experiments with this substance illustrate the important rôle which it doubtless plays. A single feeding of either the specific polysaccharide, or of the organisms, elicits the increased resistance. In some of the experiments with the former, the degree of protection and the percentage of animals immunized are so like those obtained when the entire cell is fed, as to be almost indistinguishable from them. There is a marked similarity with regard to the duration of the protection as well. Moreover, when the effect of ingesting the specific substance has worn off, an additional feeding once more brings it out. This is exactly what happens when the pneumococci are administered.

Resistance to the intestinal enzymes became an obvious property of the active constituent of the pneumococcus when immunity was obtained by feeding this organism. Other characteristics assigned to the substance were (a) its solubility in and resistance to, bile salt solutions, (b) resistance to heating at 60°C. for 1 hour in the presence of small amounts of acid, (c) type specificity, (d) considerable destruction when heated at 80°C. for 2 hours in the presence of the acid of the culture,¹⁹ (e) ready passage through the Berkefeld filter, (f) resistance to N/12 HCl at room temperature for 3 hours and (g) resistance to

¹⁸ It has been shown elsewhere that an occasional rat is protected 24 hours after feeding and vaccine.

¹⁹ The pH of the glucose-meat extract culture is approximately 4.5.

desiccation. The last two have been examined more carefully in the present work and have been confirmed. These properties are consistent with the known properties of the specific substance.

The failure to immunize mice by feeding the soluble specific substance, as opposed to the fair amount of success obtained with the whole or dissolved organism, may be considered as evidence for the presence of a second substance which is active. The partial loss of immunizing action sustained by the sodium glycocholate solution of pneumococcus on standing, may possibly be interpreted in the same way. An alternative explanation which would account for both of these observations, requires a change in the intramolecular configuration of the specific substance during its isolation and purification, and on standing in solution, accompanied by the retension of the precipitin reaction and a diminution in immunizing activity. The new form would supposedly immunize rats less well than the unaltered polysaccharide contained in the intact or freshly dissolved cell, and in the case of mice which are protected less regularly than rats by either of the latter means, would confer no protection. The absence of such a change in the whole organisms treated with $N/12$ HCl for 24 hours at room temperature could be ascribed to the fact that the rearrangement takes place more readily when the specific substance is separated from the cell. Such an assumption can be tested by determining the optical activity of a solution of purified Type I polysaccharide (presumably already partly changed) at intervals, and correlating it with the minimum quantity necessary to produce immunity when fed to rats, since a change in optical rotation is generally ascribed to a change in internal configuration of the molecule. It is interesting to note that Heidelberger, Goebel and Avery (3) have found that a sample of the specific substance of Type III pneumococcus with an initial $[\alpha]_D$ of -34.1° showed a drop in alkaline solution to -21.2° with a return to -36.0° on acidification.

SUMMARY

1. Feeding the purified soluble specific substance of Type I pneumococcus protects rats against an intraperitoneal injection of the virulent organism.
2. This increased resistance resembles that obtained when the intact

(dead) or dissolved bacteria are fed, as follows: (a) one feeding is sufficient, (b) the interval between the feeding and the appearance of the immunity is the same, (c) the duration is approximately the same, (d) when the immunity is exhausted it can be renewed by a new feeding, (e) the immunizing action is type-specific.²⁰

3. The differences between the effects of feeding the purified specific substance and the intact or dissolved organism to rats, appear to be quantitative rather than qualitative, the proportion of animals protected and the height of the immunity being generally, though not always, less in the case of the former.

4. In contrast to the immunizing action which the soluble specific substance possesses when administered to rats, feeding it to mice failed to protect them. Neither were mice definitely immunized by parenteral administration.

5. A sodium glycocholate solution of *Pneumococcus* Type I lost part of its immunizing activity on standing for 1 year.

6. The failure to immunize mice and the loss of activity of the bile salt solution of pneumococcus, on standing, are discussed in terms of (a) the possible presence of a second cell constituent which is active by mouth, and (b) a possible intramolecular change in the type-specific polysaccharide associated with a loss of immunizing action while retaining the precipitin reaction.

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²⁰ In the paper, "The rôle of the soluble specific substance in oral immunization against *Pneumococcus* Types II and III," in the next number, it is shown that feeding of Type I specific substance does not protect against Types II or III pneumococcus.

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